University of Alberta

Modulation of Apoptotic Signaling Pathways, Intracellular Localization and Secretion of Proteins by Fatty Acylation

by

Gonzalo Luis Vilas

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For Valeria,
I love you

'If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things.'

René Descartes
(1596- 1650)

Abstract

While the basic biological function of a protein is determined by its amino acid sequence, its activity, location, structure, function, stability as well as the interactions with other biomolecules are often determined by specific modifications at a particular amino acid residue. These modifications can be co- or post-translational and include phosphorylation, glycosylation, ubiquitination, methylation, acetylation and lipidation among over 200 different types.

Protein lipidation is the covalent attachment of a lipid moiety to a protein. Based on the identity of the attached lipid, protein lipidation is subdivided into four major categories: glypiation, cholesteroylation, prenylation, and fatty acylation.

Protein fatty acylation is the modification of proteins by fatty acids. The two most common fatty acid moieties present on proteins are the 16-carbon saturated fatty acid palmitate and the 14-carbon saturated fatty acid myristate. As such, eukaryotic proteins are said to undergo mainly two different types of fatty acylation events: palmitoylation and myristoylation. These processes greatly alter the physical, functional and localization properties of the modified proteins.

The mechanisms underlying the fatty acylation-dependent regulation of cellular signalling pathways and trafficking of acylated proteins remain unclear and therefore required further insights to understand of them better.

We sought to determine the importance of palmitoylation in apolipoprotein B mediated lipoprotein particle assembly and secretion as well as the role of post-translational myristoylation of caspase-cleaved PAK2 during apoptosis.

We demonstrate for the first time, the involvement of protein palmitoylation as an active signal of the ER protein sorting and folding process in a secreted palmitoylated protein, apoB.

We also identified a new post-translationally myristoylated caspase-cleaved protein: C-t-PAK2 and showed that this modification plays an important role in the capacity of C-t-PAK2 to regulate the cytoskeletal structure during apoptosis.

Overall, the results presented here demonstrate that fatty acylation of proteins plays critical roles in membrane association, targeting, localization and lead to a better understanding of the regulation of various cellular processes.

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List of Symbols and Abbreviations

$\Delta_{\Psi m}$	Mitochondrial membrane potential
a.u.	Arbitrary units
ADP	Adenosine 5'-(trihydrogen diphosphate)
AIDS	Acquired immunodeficiency syndrome
AIF	apoptosis inducing factor
Akr1	Ankyrin repeat-containing protein 1
ANT	Adenine translocator
Apaf-1	Apoptotic protease activating factor-1
apoB	Apolipoprotein B
APT1	Acyl-protein-thioesterase1
APT1	Apoptosis antigen 1
ARF1	ADP-ribosylation factor 1
ATP	Adenosine 5'-triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bap31	B Cell Receptor Associated Protein, 31kDa
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell CLL/lymphoma 2
Bcl-B	Bcl-2-like 10 protein
Bcl-G_L	Bcl-Gonad long

Bcl-W	Bcl-2-like 2
Bcl-Xl	Bcl-2-related gene long isoform
Bcl-Xs	Bcl-2-related gene short isoform
Bfk	Bcl-2 family kin
Bfl-1/A1	Bcl related gene BFL1/ protein A1
BH	Bcl-2 homology domain
BID	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
Bmf	Bcl-2-modifying factor
BNIP3	Bcl-2/adenovirus E1B 19-kD protein-interacting protein 3
Bok	Bcl-2-related ovarian killer
Boo/Diva	Bcl-2-like 10
BSA	Bovine serum albumin
CAD	Caspase-activated DNAse
cAMP	3'-5'-cyclic adenosine monophosphate
CARD	Caspase recruitment domain
CCR5	Chemokine receptor CC motif 5
CD95	Tumor necrosis factor receptor superfamily, member 6
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	Cycloheximide
c-IAP-1	Cellular inhibitor of apoptosis 1

c-IAP-2	Cellular inhibitor of apoptosis 2
CIP	Calf intestinal alkaline phosphatase
CLX	Calnexin
CM	Chylomicrons
CRIB	Cdc42/Rac interactive binding
CRT	Calreticulin
CVD	Cardiovascular diseases
Cyp-D	Cyclophilin D
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DcR3	Decoy receptor 3
DD	Death domain
DED	Death effector domain
DFF	DNA fragmentation factor
DiI-LDL	Low-Density Lipoprotein from human plasma, DiI complex
DISC	Death receptor-induced signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent Protein Kinase
DR3	Death receptor-3
DR4	Death receptor 4

DR5	Death receptor 5
DR6	Death receptor 6
Drp1	Dynamin-related protein 1
DSP	Dithiobis(succinimidylpropionate)
DTT	Dithiothreitol
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Engineered green fluorescent protein
EGTA	Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N',N'-TetraAcetate
endo H	Endoglycanase H
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
ETA	Human endothelin receptor A
FADD	Fas-associated death domain protein
FADD-DD	FADD-death domain
FADD-DED	FADD-death effector domain
FasL	Fas ligand
FBS	Fetal bovine serum
FCCP	Para-trifluoromethoxy carbonyl cyanide
FDB	Familial ligand defective apoB-100
FITC	Fluorescein isothiocyanate
FTase	Farnesyl protein transferase

GAA1	GPI anchor attachment protein 1
GAD65	Glutamic acid decarboxylase-65
GFP	Green fluorescent protein
GGTase-1	Geranylgeranyl prenyltransferase-1
GGTase-2	Geranylgeranyl prenyltransferase-2
GNAT	GCN5-related N-acetyl-transferases
GNT	Giantin
GPCR	G protein coupled receptor
GPI	Glycosyl phosphatidyl inositol
GPI8	GPI protein 8
GPI-specific PLC	Glycosyl phosphatidyl inositol- specific phospholipase C
HA	Hemagglutinin
HDJ2	Human DnaJ homolog 2
HEPES	N-Cyclohexyl-2-aminoethanesulfonic acid
Hh	Hedgehog signalling protein
HIP14	Huntingtin interacting protein 14
HIV-1 Gag	Human immunodeficiency virus type 1 group specific antigen
HIV-1 Nef	Human immunodeficiency virus type 1 negative factor
HKII	Hexokinase II
HMA	2-hyroxy-myristic acid
H-Ras	Harvey rat sarcoma viral oncogene homolog
HRP	Horse radish peroxidase
HS	Horse serum

ICAD	Inhibitor of caspase-activated DNAse
Icmt	Isoprenylcysteine carboxyl methyltransferase
IDL	Intermediate density lipoproteins
JNK	c-Jun N-terminal kinase
K-Ras	Kristen rat sarcoma viral oncogene homolog
LDL	Low-density lipoproteins
LH/CGr	Lutropin/Choriogonadotrophin receptor
MARCKS	Myristoylated alanine-rich C-kinase substrate
MBOAT	Membrane-bound O-acyltransferase
Mcl-1	Myeloid cell leukemia 1
MS	Mass spectrometry
mtCK	Mitochondrial creatine kinase
Mtd	Matador protein
MTP	Microsomal triglyceride transfer protein
NCAM	Neural Cell Adhesion Molecule
NCS-1	Mammalian neuronal calcium sensor-1
NEB	New England Biolabs
NF-κB	Nuclear Factor kappa B
NHEJ	Non-homologous end joining
Nix	Nip3-like protein X
NMT	Myristoyl-CoA: protein N-myristoyltransferase
NP40	Nonidet P-40
N-Ras	Neuroblastoma Harvey rat sarcoma viral oncogene homolog

OBCAM	Opioid Binding Cell Adhesion Molecule
Omi/HtrA2	Mammalian homologue of high temperature requirement A2
p17MA	Human immunodeficiency virus type 1 matrix protein
$p56^{lck}$	Lymphocyte cell-specific protein-tyrosine kinase
$p59^{fyn}$	Proto-oncogene tyrosine-protein kinase Fyn
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated protein kinase
PARP	Poly-(ADP-ribose) polymerase
PAT	Thiol-directed protein acyltransferase
PBD	p21-binding domain
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PIG-S	Phosphatidylinositol-glycan biosynthesis protein, class S
PIG-T	Phosphatidylinositol-glycan biosynthesis protein, class T
PI-PLC	Phosphatidylinositol-specific phospholipase C
PI-PLD	Phosphatidylinositol-specific phospholipase D
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
Pr55gag	Human immunodeficiency virus type 1 group specific antigen precursor

pRb	Retinoblastoma protein
PSD-95	Postsynaptic density protein of 95 kDa
PTP	Permeability transition pore
PUMA	p53-upregulated modulator of apoptosis
PVDF	PolyVinylidine Di-Fluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RAIDD	RIP-associated ICH-1/CED3 homologous protein with DD
Rapl A	Rap1-binding molecule A
Rapl B	Rap1-binding molecule A
Rce1	Ras-converting enzyme 1
Rho A	Ras homolog gene family, member A
Rho B	Ras homolog gene family, member B
RIP	Receptor interaction protein
R-MLC	Regulatory myosin light chain
ROCK	Rho associated coiled coil –containing protein kinase
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulphate
Shh	Sonic Hedgehog
Smac/Diablo	second mitochondria- derived activator of caspase/direct IAP-binding protein with low PI
SNAP-25	Synaptosome-associated protein of 25000 dalton
Spike	Small protein with inherent killing effect
Src-NRTK	Rous sarcoma virus tyrosine kinase homolog-non receptor tyrosine kinase

Ste20	Serine/threonine-protein kinase STE20
STS	Staurosporine
TCA	Trichloroacetic acid
TEMED	N,N,N',N',-tetramethylenediamine
TNF	Tumour necrosis factor
TNF-R1	Tumor necrosis factor-receptor 1
TNF-α	Tumour necrosis factor alpha
TRADD	Tumour necrosis factor receptor- associated death domain protein
TRAF2	Tumour necrosis factor receptor-associated factor-2
TRAIL-R1	TNF-related apoptosis inducing ligand receptor-1
TRAIL-R2	TNF-related apoptosis inducing ligand receptor-2
TRAMP	TNF-receptor-related apoptosis-mediating protein
TSHr	Human thyrotrophin receptor
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
VDAC	Voltage-dependent anion channel
VLDL	Low-density lipoproteins
VP4	Poliovirus nucleocapsid 4
Wnt	Wingless
WT	Wild type
XIAP	Inhibitor of apoptosis
Yes-NRTK	Proto-oncogene tyrosine-protein kinase-non receptor tyrosine kinase

CHAPTER 1

INTRODUCTION

1.1 Protein Lipidation

In order to maintain morphology, integrity and homeostasis cells rely on the activity of proteins. While the basic biological function of a protein is determined by its amino acid sequence, its activity, location, structure, function, stability as well as the interactions with other biomolecules are often determined by specific modifications at a particular amino acid residue. These modifications can occur co- or post-translationally and consist of over 200 different types of alterations including phosphorylation, glycosylation, ubiquitination, methylation, acetylation and lipidation (Jensen, 2006).

In particular, protein lipidation is the covalent attachment of a lipid moiety to a protein. This process greatly alters the physical, functional and localization properties of the modified protein (Magee and Seabra, 2005; Resh, 1999; Resh, 2004). Protein lipidation is subdivided into four major categories: glypiation, cholesteroylation, prenylation, and fatty acylation based on the identity of the lipid attached to the protein (Fig. 1.1) (Resh, 2004).

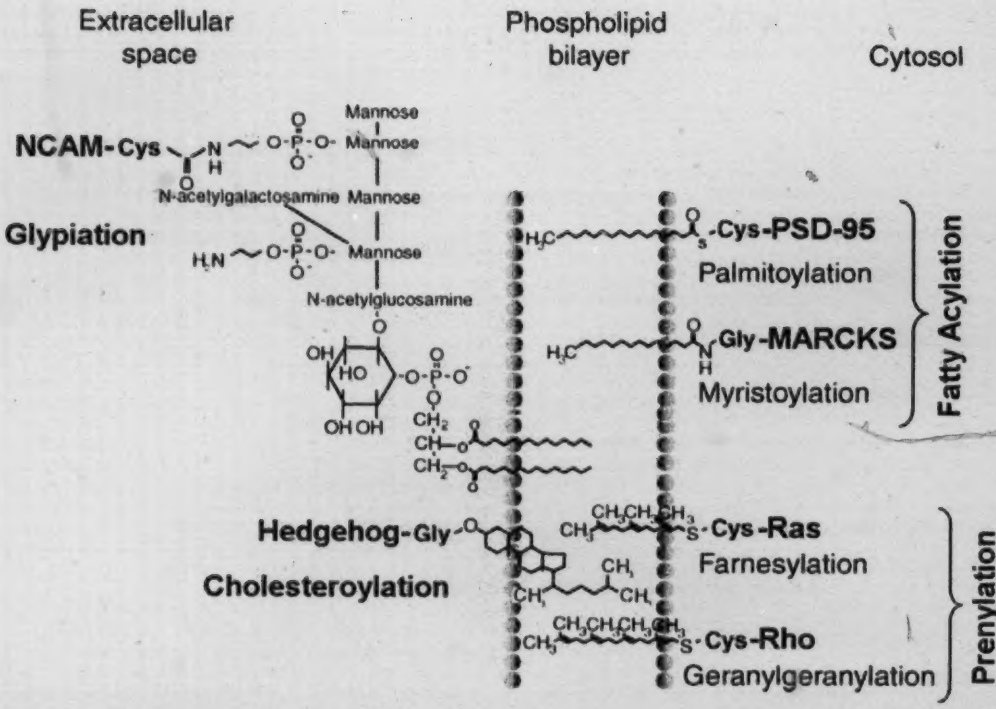


Figure 1.1. **Classes of protein lipidation.** Schematic representation of the four major categories of lipid anchors by which cellular proteins are attached to membranes. Adapted from Ferri *et al.* (Ferri et al., 2005).

1.1.1 Glypiation

Glypiation involves the modification of proteins by a complex glycosyl phosphatidyl inositol (GPI) moiety. The GPI-modified protein family is highly heterogeneous and includes enzymes (e.g. alkaline phosphatase, acethylcholinesterase, lipoprotein lipase), adhesion molecules (e.g. Neural Cell Adhesion Molecule, NCAM, Opioid Binding Cell Adhesion Molecule, OBCAM), receptors (e.g. folate receptor, fibronectin receptor) and surface antigens (e.g. cerebroglycans, CD48, pathogen *T. Brucei* surface glycoprotein) (Chatterjee and Mayor, 2001). The precursor GPI moiety is synthesized at the surface of the ER, transferred into the lumen, further modified and attached to the carboxy-terminus of various proteins after a long hydrophobic stretch of amino acids. The core motif of the GPI anchor consists of ethanolamine phosphate, trimannoside, glucosamine and inositol phosphate (Ferri et al., 2005; Ikezawa, 2002). The GPI moiety is bound to the carboxy-terminus of a protein via the ethanolamine head (Englund, 1993). The attachment of the GPI moiety to the carboxy-terminus (ω-site) of the polypeptide occurs by a transamidation reaction within the endoplasmatic reticulum following proteolytic cleavage of a carboxy-terminal pro-peptide from the protein (Mayor and Riezman, 2004). This process anchors the modified proteins to the lumenal side of membranes of the secretory and endocytic pathways as well as to the exoplasmic leaflet of the plasma membrane (Englund, 1993).

In mammalian cells, the GPI anchor attachment protein 1 (GAA1), GPI protein 8 (GPI8), as well as the class S and class T Phosphatidylinositol-glycan

biosynthesis proteins (PIG-S and PIG-T) form the GPI-transamidase complex required for the formation of a carbonyl intermediate between the transamidase and precursor protein and the transference of the GPI moiety (Ikezawa, 2002; Ohishi et al., 2000). Based on computational analysis of 155 GPI-anchored proteins and site-directed mutagenesis studies performed on model proteins, a carboxy-terminal consensus sequence carrying the GPI-modification signal has been proposed. The predicted GPI-modification consensus sequence consists of four regions: 1) an unstructured linker region of 11 residues from ω-11 to ω-1, which is flexible and polar; 2) a region of small residues from ω-1 to ω+2 including the GPI-attachment site (ω site); 3) a spacer sequence (ω+3 to ω+8) of moderately polar regions containing hydrophobic residues, and 4) a hydrophobic tail from ω+9 to the carboxyl-terminal end (Eisenhaber et al., 1999; Eisenhaber et al., 2001; Ikezawa, 2002; Ohishi et al., 2000).

Genomic analyses suggest that GPI-anchored proteins represent, on average, about 0.5% of cellular proteins in eukaryotes (Eisenhaber et al., 2001). GPI moieties are a diverse group of structures that have a common core motif that is conserved from yeast to mammals, suggesting that this modification has a high functional importance (Ikezawa, 2002).

In mammalian cells, the GPI anchor enables proteins to relocalize to lipid rafts (Mayor and Riezman, 2004) where they often transduce signals across the bilayer resulting in several intracellular responses such as oxidative burst, Ca^{2+} influx, protein tyrosine phosphorylation, cell proliferation or inhibition of cell growth (Ferri et al., 2005). GPI-anchored proteins can also be released from the

plasma membrane by the action of endogenous hydrolases including PI-phospholipase C (PI-PLC), PI-specific phospholipase D (PI-PLD) and GPI-specific PLC (Mayor and Riezman, 2004). The anchorage provided to the protein by the GPI moiety is more stable than other lipid-based anchors such as myristoyl, palmitoyl and prenyl groups, which allow only weak transient membrane anchorage (Mayor and Riezman, 2004).

1.1.2 Cholesteroylation

Cholesteroylation consists of the modification of proteins by cholesterol. The members of the Hedgehog (Hh) signalling protein family are, so far, the only identified examples of proteins known to be covalently modified by cholesterol (Ferri et al., 2005; Mann and Beachy, 2004). Newly synthesized Hh proteins are subjected to a series of post-translational modifications, including cholesteroylation, that occur along their trafficking through the secretory pathway. These modifications result in the formation of an active signalling protein on the cell surface. After entering the secretory pathway, an amino-terminal signal sequence is removed and the carboxy-terminal domain of Hh auto-catalyzes the internal cleavage between G-C residues of a G-C-F tripeptide that is conserved in all family members (Lee et al., 1994; Porter et al., 1995). The terminal glycine of the resulting amino-terminal cleavage product is the acceptor of the cholesterol moiety. After the covalent attachment of the cholesterol molecule to the glycine residue, the Hh protein signalling domain becomes active. The carboxy-terminal domain of the Hh precursor has no other

known function but to mediate the auto-catalytic reaction (Ferri et al., 2005; Mann and Beachy, 2004). In addition to cholesteroylation, Hh is also modified at its amino-terminal cysteine residue by palmitoylation via an atypical amide bond.

Cholesterol alone is able to anchor Hh proteins to membranes with significant strength (Peters et al., 2004). Its membrane anchoring ability is comparable with dual lipidation motifs such as double geranylgeranylation or S-palmitoylation plus S-farnesylation found in other lipidated proteins (Peters et al., 2004). Importantly, many other cholesterol-modified proteins have been detected in mammalian cells that still need to be identified (Mann and Beachy, 2004; Porter et al., 1996). This observation suggests that cholesterol modification of polypeptides may also be employed as a means of directing proteins, other than Hedgehog, to membranes or other hydrophobic targets (Mann and Beachy, 2004; Porter et al., 1996).

1.1.3 Prenylation

Prenylated proteins are modified by one of two types of isoprenoid lipids, either the 15-carbon farnesyl or the 20-carbon geranylgeranyl moieties (Lobell, 1998). Isoprenoids are polymers of the common five-carbon building unit isopentenyl diphosphate and its isomer dimethylallyl diphosphate, also commonly referred to as isoprene units (McTaggart, 2006). Farnesyl isoprenoid is derived from isopentenyl diphosphate, while geranylgeranyl pyrophosphate is derived directly from farnesyl pyrophosphate to which an extra isoprenoid unit has been added (McTaggart, 2006; Rodriguez-Concepcion and Boronat, 2002).

Three prenyltransferases are expressed in mammalian cells: farnesyl protein transferase (FTase), geranylgeranyl prenyltransferase-1 (GGTase-1) and geranylgeranyl prenyltransferase-2 (GGTase-2) (McTaggart, 2006). FTase catalyzes the transfer of farnesyl phosphate onto a cysteine residue in the carboxy-terminal tetra-peptide motif C-a-a-X (where "C" is a cysteine residue, "a" is an aliphatic amino acid, and X is the carboxy-terminal amino acid residue). Proteins presenting the C-a-a-X motif whose carboxy-terminal amino acid is serine, methionine, glutamine, or alanine are FTase substrates. Such substrates include H-Ras, N-Ras, K-Ras, paralemmin A, HDJ2 and PTP-CAAX/ PRL tyrosine phosphatases (McTaggart, 2006).

GGTase-1 catalyzes the transfer of the 20-carbon isoprene moiety geranylgeranyl to proteins that contain a C-a-a-L (where "L" is a leucine residue) carboxy-terminal motif. Among the proteins modified by GGTase-1 are Rac1, CDC42, Rho A, Rho B, 2', 5' Oligoadenylate synthetase 1, Rapl A and Rapl B (Roskoski, 2003). Of note, both FTase and GGTase are $\alpha\beta$ heterodimers with a common α subunit (McTaggart, 2006; Resh, 2004).

The third prenyltransferase, GGTase-2, specifically catalyzes the transfer of geranylgeranyl moieties onto the carboxy-terminus of the members of the Rab family of small GTPases. This family of proteins are involved in the regulation of intracellular vesicular trafficking and generally their carboxy-terminal motif contains two cysteine residues (X-X-C-C, X-C-X-C, or C-C-X-X) (Basso et al., 2006; Ferri et al., 2005; McTaggart, 2006; Roskoski, 2003).

The isoprenoids that these three prenyltransferases transfer to the cysteine residues at or near the carboxy-terminus of their substrates, are attached to them through a stable thioether bond. This transfer process results in an irreversibly modified protein and the isoprenyl moiety does not appear to turnover (Roskoski, 2003).

Upon lipidation by FTase and GGTase1, the newly prenylated proteins further undergo two consecutive post-translational modifications. These modifications consist of the removal of the last three amino acids from the protein (the -a-a-X motif) in a reaction catalyzed by the prenylprotein-specific endoprotease, Ras-converting enzyme 1 (Rce1) (Bergo et al., 2004) and the methylation of the newly exposed carboxy-terminal isoprenylcysteine by the isoprenylcysteine carboxyl methyltransferase (Icmt) (Sinensky, 2000). Prenylation of C-a-a-X proteins is vital to eukaryotic cells. In fact, between 0.5 and 2% of total cellular mammalian proteins are estimated to undergo geranylgeranylation or farnesylation (McTaggart, 2006), two important post-translational modifications that are required for their proper trafficking, targeting to membranes and function (Magee and Seabra, 2005).

1.1.4 Fatty acylation

Protein fatty acylation is the modification of proteins by fatty acids. The two most common fatty acid moieties present on proteins are the 16-carbon saturated fatty acid palmitate and the 14-carbon saturated fatty acid myristate (Resh, 1999). As such, eukaryotic proteins are said to undergo mainly two

different types of fatty acylation events: palmitoylation and myristoylation (Linder and Deschenes, 2003). Fatty acylation is in many cases involved in the modulation of protein-protein as well as lipid-protein interactions and can therefore modulate the activity (hungtitin, CPS1), localization (PSD-95, hungtitin) and conformation [Regulator of G protein signaling (RGS)] of the modified protein (Corvi et al., 2001; Huang et al., 2004; Ni et al., 2006; Yanai et al., 2006). Since protein fatty acylation and the way in which the addition of palmitate and myristate alter the proper sorting and conformation of proteins is the focus of our investigations, this particular category of protein lipidation is reviewed more thoroughly below.

1.1.4.1 Palmitoylation

Palmitoylation is the covalent attachment of palmitate to a protein. Based on the type of linkage between the palmitoyl moiety and the acceptor amino acid residue, palmitoylation can be sub-divided into N- and S- palmitoylation, depending on whether the covalent linkage occurs via an amide or a thioester bond, respectively (Fig. 1.2) (Resh, 1999).

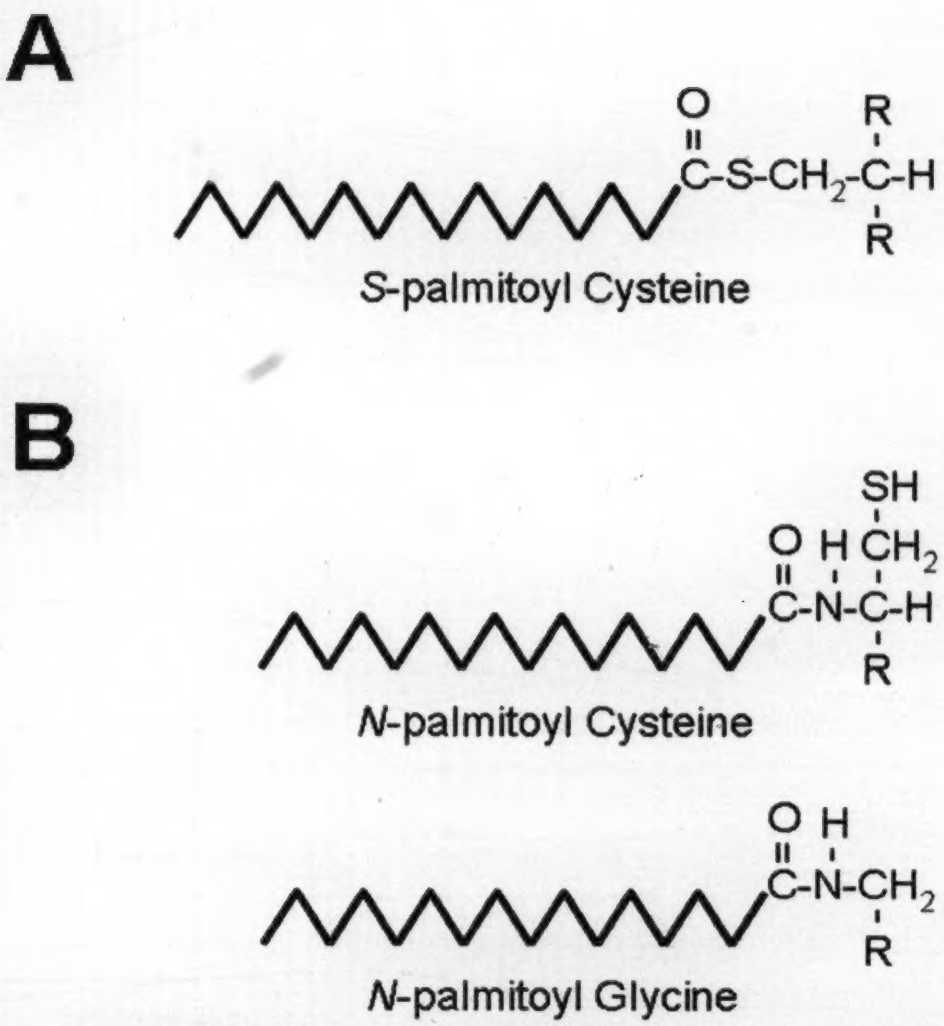


Figure 1.2. **Structures of the different palmitoyl modifications found at or near the amino termini of proteins.** In S-palmitoylation the palmitoyl moiety is bound to the cysteine residue via a thioester bond (A). In N-palmitoylation the palmitoyl moiety is bound either a cysteine or a glycine residue via an amide bond (B). R denotes the continuation of the polypeptide backbone.

Like other types of lipid modifications of proteins, protein palmitoylation promotes membrane association of proteins that otherwise would remain soluble or poorly attached to membranes. Palmitoylation has been shown to occur on a wide variety of proteins, including peripherally associated, secreted and integral membrane proteins (Ferri et al., 2005; Resh, 1999). However, in addition to serving as a membrane anchor, palmitoylation has also been shown to directly regulate protein trafficking, organelle inheritance, and vesicle fusion (Bijlmakers and Marsh, 2003; el-Husseini Ael and Bredt, 2002; Silvius, 2002). In addition, modification of proteins with palmitate has also been shown to influence the lateral distribution of proteins on the plasma membrane by targeting them to 'lipid rafts' (Resh, 2004). Finally, *in vitro* assays have revealed the importance of palmitoylation in promoting or inhibiting protein-protein interactions and protein activities (Smotrys and Linder, 2004). Palmitoylation also works in concert with other lipid modifications of proteins and protein motifs to facilitate targeting to the appropriate cellular destination through mechanisms that are just beginning to be defined (McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001).

Overall, since the discovery of the covalent attachment of fatty acids to eukaryotic proteins was first described almost 35 years ago (Braun and Radin, 1969; Stoffyn and Folch-Pi, 1971), the body of knowledge acquired on the biological functions of this modification has shown that palmitoylation is an active and versatile molecular modulator of protein activity, sorting and function.

1.1.4.1.1 S-Palmitoylation

In S-palmitoylation, the sixteen carbon fatty acid palmitate is transferred from a palmitoyl-CoA molecule to variably located cysteine residues of a protein (Resh, 1999; Smotrys and Linder, 2004). This transfer reaction proceeds via a thioester bond in a post-translational and reversible fashion. Palmitoylation, rather than S-palmitoylation or S-acylation, is currently the most utilized terminology in the literature since palmitate is the preferred fatty acid transferred onto cysteine residues of proteins (Resh, 1999; Smotrys and Linder, 2004). From now on, palmitoylation will be used to describe S-palmitoylation of cysteine residues. Typically, palmitoylation occurs at cysteine residues in proximity to a myristoylated glycine residue, a farnesylated/carboxymethylated cysteine residue, a transmembrane domain or, in some cases, on the active site cysteine residue(s) of mitochondrial enzymes (Berthiaume et al., 1994; Corvi et al., 2001; McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001; Resh, 1999; Resh, 2004; Smotrys and Linder, 2004).

The observation that a protein can undergo several cycles of palmitoylation and de-palmitoylation during its life time led to the hypothesis that palmitoylation, like phosphorylation, can act as a molecular switch that controls a variety of regulatory processes in the cell (Huang and El-Husseini, 2005; Mumby, 1997; Qanbar and Bouvier, 2003).

For example, de-palmitoylation of PSD-95, a scaffolding protein at excitatory glutamatergic synapses that organizes a postsynaptic network of signaling molecules, promotes the removal of this protein from synapses and

thus regulates the internalization of the glutamate receptor type 1 GluR1 (el-Husseini Ael and Bredt, 2002; El-Husseini Ael et al., 2002; Huang and El-Husseini, 2005). It has also been shown that the activation of the GPCR β-adrenoreceptor enhances the removal of the palmitoyl moiety from of the associated $G_{\alpha s}$ subunit and diminishes G-protein signaling (Qanbar and Bouvier, 2003). Finally, the work of Drenan *et al.* on the palmitoylation-depalmitoylation cycles of R7BP, a neuronal protein that binds R7-Gβ5 complexes, has shown that this process regulates the shuttling of the R7BP-R7-Gβ5 complexes from the plasma membrane to the nucleus regulating transcriptional control (Drenan et al., 2005). These examples support the idea that dynamic protein palmitoylation turnover is a widely used mechanism to control several signaling pathways. Interestingly, certain proteins such as synaptotagmin I and SNAP-25 present a much slower palmitate turnover. In the case of these proteins, palmitoylation probably functions as a structural signal for correct targeting and assembly of elements involved in neurotransmitter release (Huang and El-Husseini, 2005).

In the first half of the past decade, it was reasoned that since the formation of a palmitoyl-thioester bond on polypeptides was shown to occur spontaneously *in vitro* only in the presence of palmitoyl-CoA and at neutral pH (Bizzozero et al., 2001; Duncan and Gilman, 1996; Veit et al., 2000; Veit et al., 2001), protein palmitoylation also probably occurred in a similar, non enzymatic, fashion *in vivo* (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996; Quesnel and Silvius, 1994). This notion was also supported by two

observations: (a) although several biochemical studies were successful in enriching a protein-acyl-transferase (PAT) activity, they didn't succeed in pinpointing the protein responsible for the reaction (Berthiaume et al., 1995; Das et al., 1997; Kasinathan et al., 1990) or found proteins that eventually turned out to be false positives (Liu et al., 1996), (b) there isn't a clear consensus sequence on palmitoylated proteins that can facilitate a prediction of such modification. In fact, most palmitoylated proteins only share certain common features such as a membrane targeting sequence in the vicinity of the target cysteine residues that consists of basic amino acidic residues, adjacent lipid anchors or trans-membrane domains (Bijlmakers and Marsh, 2003).

However, since the discovery of two different types of PAT enzymes this concept has been challenged (Mann and Beachy, 2004; Mitchell et al., 2006). Studies have shown that such PATs not only carry out the thioesterification of the cysteine residue with palmitate (Linder and Deschenes, 2003; Linder and Deschenes, 2004) but also they do so with other saturated and unsaturated fatty acyl groups as well (Liang et al., 2002; Liang et al., 2001; O'Brien et al., 1987). Therefore, it has been proposed to re-name this type of enzyme thiol-directed protein acyltransferases and keep the same acronym: PAT (Mitchell et al., 2006). Based on the lack of sequence homology and the distinction between intracellular versus secreted substrates, it appears that at least two distinct classes of PATs have evolved.

The first class of PATs acylate intracellular proteins and share a domain referred to as the DHHC-CRD domain, a conserved aspartate-histidine-

histidine-cysteine motif with a cysteine-rich domain (Mitchell et al., 2006). Studies have presented evidence suggesting that the DHHC domain is directly involved in the palmitate transfer reaction (Mitchell et al., 2006). Several members of the DHHC protein family have been demonstrated to possess PAT activity, including Erf2/Erf4, the yeast heterotrimeric PAT that palmitoylates prenylated Ras, Akr1, the PAT for yeast casein kinase I (Lobo et al., 2002; Roth et al., 2002) and HIP14 (Singaraja et al., 2002). HIP 14 is a neuronal protein which showed PAT activity for the amino-terminal fragment of Huntington (htt) (1–548), SNAP-25, PSD-95, GAD65, and synaptotagmin I (Huang et al., 2004; Yanai et al., 2006). So far, all the eukaryotic genomes examined to date present genes encoding DHHC proteins. Their numbers vary from 1 to 8 in fungi to more than 20 in metazoans (Mitchell et al., 2006; Roth et al., 2006). In the few cases where they have been studied, DHHC proteins were found to be expressed in different types of tissues and to be present in different sub cellular locations (Saitoh et al., 2004; Swarthout et al., 2005; Uemura et al., 2002). This suggests that intracellular PATs play diverse roles in cells and present different substrate specificity and regulation.

Utilizing a proteomic approach, Roth et al. characterized the palmitoyl proteome of the yeast *Saccharomyces cerevisiae* and analyzed the substrates specificities of different DHHC-containing PATs present in this organism (Roth et al., 2006). In this work they found that the DHHC PATs have both discrete and overlapping specificities that together mediate the diverse palmitoylations that occur within the cell (Roth et al., 2006).

The second class of PATs acts on proteins that are modified in the lumen of the secretory pathway. Very little is known about this type of PATs and the role they have during the secretory process of palmitoylated proteins. Genetic evidence from *Drosophila melanogaster* suggests the existence of enzymes that modify secreted morphogens of the Wnt and Hedgehog families (Mann and Beachy, 2004). Skinny hedgehog is required for the palmitoylation of Hedgehog (Chamoun et al., 2001) and Spitz (Miura et al., 2006) at amino-terminal cysteine residues. Porcupine is required for the modification of Wnt proteins (Zhai et al., 2004). Molecular characterization of the Skinny hedgehog and Porcupine genes revealed that they encode proteins belonging to the membrane-bound O-acyltransferases (MBOAT) family of enzymes (Bosson et al., 2006), some of which are known to catalyze esterification reactions involving, principally, lipids and other relatively small molecules (Hofmann, 2000). Skinny hedgehog and Porcupine share limited sequence homology with O-acyltransferases (Hofmann, 2000) but lack the DHHC domain that is found in intracellular PATs (Mann and Beachy, 2004).

In addition to the palmitoylation reactions mediated by intracellular PATs, the cycling of palmitate involves the removal of the palmitoyl moiety by protein palmitoyl thioesterases that must be present in the appropriate sub-cellular region and be able to be induced to mediate depalmitoylation under specific conditions (Bijlmakers and Marsh, 2003). So far, only one acyl-protein-thioesterase (APT1) that depalmitoylates Gα subunits, Ras and eNOS *in vitro*, and $G_{\alpha s}$ *in vivo*, has been described (Duncan and Gilman, 1998; Yeh et al.,

1999). APT1 is widely conserved from yeast to humans and was purified from rat liver cytosol based on its ability to remove palmitate from $G_{\alpha i}$ (Duncan and Gilman, 1998). APT1 is a 29 kDa protein that was previously characterized as having lysophospholipase activity toward palmitoylated glycerol-3-phosphocholine (Sugimoto et al., 1996). Studies have shown that APT1 prefers activated proteins as substrates for its palmitoyl-thioesterase activity. G_{α} was proven to be a better substrate for APT1 in the absence of $G_{\beta\gamma}$ or when activated with AlF_4^- to release $G_{\beta\gamma}$ from the heterotrimeric complex (Duncan and Gilman, 1998; Duncan and Gilman, 2002). Similarly, APT1 seems to be more efficient at removing the palmitoyl moiety from eNOS *in vitro* in the presence of the eNOS-activating Ca^{2+}-calmodulin complex (Yeh et al., 1999). The same appears to be true for Ras proteins, since the inactive isoform H-Ras shows a slower palmitate turnover *in vivo* than the constitutively active oncogenic isoforms (Baker et al., 2003). As mentioned at the beginning of this section, activation of some signalling proteins correlates with increased palmitate turnover (James and Olson, 1989; Mundy and Warren, 1992; Patterson and Skene, 1999; Veit et al., 2000). The observation that APT1 prefers activated proteins as substrates for its palmitoyl-thioesterase activity is consistent with those observations. Based on the diversity of activated substrates susceptible to APT1 activity, it appears that APT1 does not recognize a specific protein sequence but rather a conformational change (or dissociation of a binding partner) at or near the palmitoyl moiety that may make the modified amino acidic residue more accessible for cleavage, independently of the protein sequence context. Another

possibility relates to the proximity of APT1 to its substrate. As mentioned, APT1 was purified from cytosol, but its substrates are found attached to membranes. It is unknown whether APT1 is recruited to these membranes in response to a signal or if the abundance of APT1 is such that there is enough protein present near membranes to de-palmitoylate its target(s).

Palmitoylation has been proven to be a dynamic modification that can reversibly alter the function and localization of various proteins. It is clear that many aspects of this modification still remain to be explained. The original contribution of the palmitoyl moiety was believed to simply anchor the modified protein to a membrane but this cannot be true of all proteins since several membrane proteins with multiple transmembrane domains are also palmitoylated. As such, palmitoylation is probably more important for trafficking between membrane compartments or membrane sub-domains within the same membrane than simply serving as a stable membrane anchor. A key area for future investigation will be to determine how protein-bound palmitate cooperates with other proteins and membrane lipids to direct proteins to their appropriate membrane compartment and regulates their localization and in some cases their secretion (e.g. Hh, Wnt, apoB).

1.1.4.1.2 N-palmitoylation

In N-palmitoylation a palmitoyl moiety is irreversibly added to an amino-terminal cysteine or glycine residue via an amide bond. N-palmitoylation was first described to occur on the amino-terminal signalling domain of Sonic

Hedgehog (Shh) (Pepinsky et al., 1998). Shh is a member of the Hedgehog signalling protein family, and is involved in diverse embryonic induction events, including induction of floor plate, establishment of ventral polarity within the central nervous system, and proper anterior-posterior patterning of the developing limb (Rajala, 2005). N-palmitoylation of the Shh protein occurs in the lumen of the endoplasmic reticulum and on the amino-terminal cysteine residue that is exposed in the signalling domain of the molecule after the cleavage of the signal sequence (Mann and Beachy, 2004). Apparently, the efficiency and specificity of this modification is regulated by the initial addition of cholesterol to the carboxy-terminus of the signalling domain, since the level of acylation is reduced and the type of added fatty acids vary when the Shh protein is produced from a truncated construct lacking the processing peptide sequence (Taipale et al., 2000). N-palmitoylation of Shh further increases the hydrophobicity and the signal potency of the Shh protein (Mann and Beachy, 2004). Genetic studies performed on the fruit fly *Drosophila melanogaster* have proven the existence of an acyl-transferase that is necessary for the transfer of a palmitate moiety to the amino-terminal cysteine of secreted morphogens of the Wnt and Hedgehog families (Mitchell et al., 2006). Skinny hedgehog (also known as Rasp, Central missing, and Sightless) has been shown to be responsible for the palmitoylation of Hedgehog (Chamoun et al., 2001) and more recently of Spitz (Miura et al., 2006) at amino-terminal cysteine residues while Porcupine was identified as the transferase that modifies Wnt.

It has been proposed that the reaction by which the amino-terminal cysteine residue of Hh proteins becomes N-palmitoylated likely occurs by conventional side chain S-palmitoylation followed by a spontaneous thiol to nitrogen (S to N) shift that results in the formation of the more energetically favourable amide bond (Taylor et al., 2001). It is thought that after the signal peptide is removed in the lumen of the endoplasmic reticulum, the primary amine present at the terminal cysteine residue of Hh proteins attacks the palmitoyl-thioester linkage generating an unstable cyclic intermediate that is then resolved into a more stable amide bond (Mann and Beachy, 2004). The observations that the S to N shift has been shown to occur *in vitro* (Muir, 2003) and that in Shh the substitution of the cysteine residue by serine blocks N-palmitoylation (Pepinsky et al., 1998), argue in favour of this mechanism.

Another variation of N-palmitoylation is found in the α subunit of the heterotrimeric G protein that activates adenylate cyclase, $G_{\alpha s}$. The amino-terminal protein sequence of this protein is M-G-C-L-G-N-S-K-T-E- (Smotrys and Linder, 2004). Like other members of the G_{α} subfamily containing a glycine and a cysteine residues at positions 2 and 3 respectively, $G_{\alpha s}$ is expected to be N-myristoylated at the glycine residue 2 after the initiator methionine residue is removed by the action of the methionyl amino peptidase and then to be S-palmitoylated at the cysteine residue 3 (Chen and Manning, 2001). However, in contradiction to what can be deduced from its taxonomical origin and amino-terminal sequence, Kleuss and Gilman reported that $G_{\alpha s}$ is indeed palmitoylated through a thioester bond at cysteine 3, but it is not N-myristoylated at glycine 2

(Kleuss and Gilman, 1997). Interestingly, in that same work the authors reported data supporting the existence of yet another covalent hydrophobic modification at or near the amino-terminus of the protein. Unfortunately, they also reported that they were not able to identify this lipophilic modification due to certain technical limitations at that time (Kleuss and Gilman, 1997). Finally, in 2003 Kleuss and Krause presented mass spectrometric data indicating that this unknown modification was indeed a second palmitoyl moiety linked to the amino terminal glycine residue 2 via an amide bond (Kleuss and Krause, 2003).

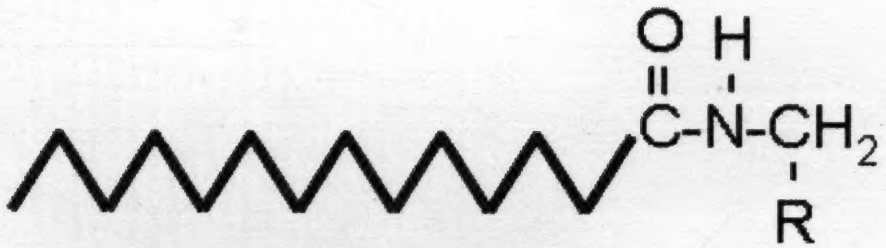
The mechanism by which the palmitate moiety is linked to the glycine residue through an amine bond remains unclear. Two possible explanations for this process have been put forward (Linder and Deschenes, 2003). In the first explanation, the cysteine residue 3 of $G_{\alpha s}$ is palmitoylated through a thioester bond and then, in a similar way as postulated for Hh, the palmitate moiety is transferred to the primary amine on the glycine residue 2 through a cyclic intermediate (Mann and Beachy, 2004). The second explanation for the N-palmitoylation of $G_{\alpha s}$ states that N-myristoyltransferase 2 (NMT 2) is capable of directly attaching a palmitate moiety to the terminal glycine residue of the protein via an amide bond. This is based on two observations. The first observation is that unlike its isoform NMT 1 (which has been shown to possess very strict substrate selectivity for myristoyl-CoA and cannot transfer palmitate (Farazi et al., 2001)), NMT 2 substrate specificity still remains unclear (Rioux et al., 2006). The second observation is that a functional characterization of both NMT1 and NMT2 in human cells using RNA interference techniques has shown

that the two isozymes have only partially overlapping functions *in vivo* (Ducker et al., 2005).

Independent of the underlying mechanism, N-palmitoylation of the glycine residue 2 of $G_{\alpha s}$ has a great impact on the $G_{\alpha s}$-mediated signal transduction at the level of adenylate cyclase regulation. Glycine 2-palmitoylated $G_{\alpha s}$ (in contrast to non-palmitoylated or cysteine 3-palmitoylated $G_{\alpha s}$) renders the cell more receptive for stimulatory inputs and less sensitive for the inhibitory stimuli that are transmitted by G protein α-subunits (Kleuss and Gilman, 1997; Kleuss and Krause, 2003). This fatty acylation process adds a new dimension of regulatory possibilities to this crucial signal transduction system.

1.1.4.2 N-Myristoylation

In N-myristoylation, the fourteen carbon fatty acid myristate via its activated myristoyl-CoA form is irreversibly transferred to a protein, via an amide bond, at specific amino-terminal glycine residues (Fig. 1.3) (Farazi et al., 2001; Resh, 1999). This modification is widespread and found in eukaryotic cells as well as viruses (Shrivastav et al., 2004). N-myristoylation is catalyzed by the enzyme myristoyl-CoA: protein N-myristoyltransferase or NMT. NMT is a member of the GCN5-related N-acetyl-transferases (GNAT) super family of proteins which catalyze the transfer of the acetyl group from acetyl-CoA to a primary amine (Dyda et al., 2000).



N-myristoyl Glycine

Figure 1.3. **Structure of the myristoyl modification found at the amino termini of proteins.** In N-myristoylation the myristoyl moiety is bound to a specific amino-terminal glycine residue via an amide bond. R denotes the continuation of the polypeptide backbone.

Nineteen NMTs have been identified from 15 different species of yeast, insects, pathogenic fungi, mammals and plants (Rundle et al., 2002). Genetic studies have established that this enzyme is essential for the development of several of them (Selvakumar et al., 2002; Yang et al., 2005). Mutations in NMT have been shown to cause recessive lethality in yeast and in *Drosophila melanogaster* (Farazi et al., 2001; Ntwasa et al., 1997). A proper NMT activity was also shown to be necessary in plants, since mutations in the gene coding for this enzyme have been proven to affect the normal growth and development of *Arabidopsis thaliana* (Qi et al., 2000; Rajala, 2005). Interestingly, it is estimated that about a hundred myristoylated proteins in lower and higher eukaryotes are involved in oncogenesis, in cellular signalling, in infectivity of retroviruses and in other virus types (Selvakumar et al., 2002; Shrivastav et al., 2005).

In humans, cows and mice two NMT isoforms are expressed. They have been termed type I and type II NMTs (Giang and Cravatt, 1998). Both type I NMT (NMT1) and type II NMT (NMT2) show a high degree of conservation across species (Farazi et al., 2001; Giang and Cravatt, 1998). Interestingly, the activity of both NMT1 and NMT2 is regulated by the binding of the heat shock cognate protein 70 (HSC70) (Selvakumar et al., 2004). NMT1 and NMT2 present the highest divergence at their amino-termini (Farazi et al., 2001). *In vitro* studies have suggested that the longer amino-terminal domain of NMT1 is involved in the association of the enzyme with the ribosome and that this extended amino-terminus is not required for the myristoyl-transferase activity of

the protein itself (Glover et al., 1997). Since NMT1 has been found to be associated to the ribosome whereas NMT2 was found in the cytosol, it is thought that the different amino-terminal domains of the two NMT isoforms may allow differential cellular localization (Farazi et al., 2001; Giang and Cravatt, 1998). This differential sub-cellular localization may in turn determine a specific sub-set of substrates for each isoform (Ducker et al., 2005). In the vast majority of cases, N-myristoylation is a co-translational process.

In order for co-translational myristoylation to take place, the initiator methionine residue has to be removed from the protein by means of the enzyme methionine-amino-peptidase (Giglione et al., 2004). Once cleavage is accomplished, the glycine residue 2 becomes the protein's amino-terminal amino acid and the myristoyl-acceptor. The requirement for a glycine residue at the amino-terminus is absolute since no other amino acid residue will be recognized by NMT as a substrate (Farazi et al., 2001; Resh, 2004). However, not all proteins with an amino-terminal glycine residue are N-myristoylated. The ability of a polypeptide to be recognized by NMT as substrate depends on the downstream amino acid sequence (Resh, 1999; Resh, 2004; Rioux et al., 2006). In contrast to the palmitoylation recognition sequence, the consensus sequence for NMT protein substrates is well defined: methionine-glycine-X-X-X-serine/threonine/cysteine. The glycine residue at position 2 is essential, a serine, threonine or cysteine residue is preferred at position 6, and lysine or arginine residue is preferred at position 7 and/or 8 (Farazi et al., 2001; Resh, 2004).

Although shorter than palmitate by only two carbons, myristate is much less hydrophobic and provides much weaker interaction with membranes (Silvius, 2002). However, myristate's specific length and structure could provide the possibility for reversible interactions with other proteins or membranes (Ferri et al., 2005; Resh, 1999). Consequently, the physicochemical properties of the myristoyl moiety allow for a variety of ways in which this modification can regulate protein/membrane and protein/protein interactions as well as protein conformational changes and activity (Taniguchi, 1999).

For example, many myristoylated proteins can be found at the plasma membrane or other intracellular membranes in eukaryotic cells. Generally, if protein myristoylation is prevented by mutating the glycine residue 2 to alanine or using a compound that blocks NMT activity such as 2-hydroxy-myristic acid (Galbiati et al., 1996; Nadler et al., 1993; Paige et al., 1990), a reduction or even a loss of membrane binding is observed (Farazi et al., 2001; Ferri et al., 2005; Matsubara et al., 2003). Such is the case for pp60v-src (Src), the transforming protein of the Rous sarcoma virus. Studies on this protein have clearly established that myristoylation is necessary for directing Src to the membrane. Src mutants that cannot be myristoylated are not able to bind to membranes and do not mediate cellular transformation (Sharma, 2004; Shoji et al., 1991). However, by itself a myristoyl moiety is not hydrophobic enough to confer stable membrane association or to confer specific membrane localization. In fact, a second signal within the myristoylated protein is necessary for efficient protein localization into membrane bilayers (Resh, 1999; Resh, 2004). In the case of

Src, the presence of a polybasic stretch of residues near the myristoyl moiety enhances its binding to membranes containing acidic phospholipids nearly 3000-fold (Hayashi et al., 2004). In membrane bound proteins, myristoylation is often accompained by adjacent or remote second membrane anchoring signals, generally one or several palmitoyl moieties (e.g. Yes-NRTK, $p59^{fyn}$, $p56^{lck}$ and eNOS) or a polybasic domain(s) (e.g. Src-NRTK MARCKS, HIV-1 Gag and HIV-1 Nef) (McCabe and Berthiaume, 1999; Resh, 1996; Resh, 1999; Resh, 2004; Resh, 2005; Wolven et al., 1997).

Evidence also suggests that the myristoyl moiety does not always protrude from a protein but nevertheless is crucial for its proper function. Crystallographic studies of several N-myristoylated proteins have revealed that the myristoyl moiety plays a structural role in stabilizing their three-dimensional conformation. For example, crystal structures obtained from recoverin (Desmeules et al., 2006; O'Callaghan and Burgoyne, 2004) and Arf-1 (Georgopapadakou, 2002) revealed binding sites for myristate in hydrophobic pockets within the proteins' three-dimensional structures. Another example of the structural role played by myristate is represented by the catalytic subunit of protein kinase A (PKA). In this subunit, myristate is positioned within a hydrophobic pocket and is required for structural and thermostability of the enzyme (Zheng et al., 1993). Finally, the non-enveloped virus Poliovirus has been shown to require the myristoylation of its VP4 protein for proper assembly. Structural studies revealed that the myristoyl moiety forms an integral part of the virion subunit structure and

mediates key hydrophobic interactions between viral subunits that stabilizes the capsid structure (Chow et al., 1987).

The relative orientation of the myristoyl moiety on the protein where it is attached is often dynamic. Interestingly, the relative moiety orientation plays an important role controlling membrane binding (Resh, 1999). It has been observed that some myristoylated proteins can exist in two different conformations. In one conformation, the protein has the myristoyl moiety sequestered inside a hydrophobic pocket and remains soluble, whereas in the alternate conformation, it undergoes a conformational change that exposes the myristoyl moiety making it available to participate in membrane binding (Resh, 2005). The conformational changes that occur between the two conformations are regulated by a mechanism called "myristoyl switch". Proteins that are regulated by a myristoyl switch typically exhibit reversible membrane binding. The events that trigger the switch can be classified into three general categories: ligand binding, electrostatic, and proteolysis (Ferri et al., 2005; Resh, 1999).

In myristoyl ligand switch, the binding of a stimulatory ligand triggers a conformational change that regulates exposure of the myristate moiety. In one conformation, myristate is sequestered within a hydrophobic cleft of the protein. In the alternative conformation, myristate is "flipped out" and promotes membrane binding (Ferri et al., 2005; Resh, 1999). Mammalian neuronal calcium sensor-1 (NCS-1), a Ca^{2+}-binding protein, is expressed in neurons and neuromuscular junctions and plays an important role in the modulation of neurotransmitter release and in the phosphatidyl inositol signalling pathway.

When Ca^{2+} binds to this protein, it induces a conformational change leading to the exposure of a buried amino-terminal myristoyl moiety (Ames et al., 1997; Jeromin et al., 2004; Zozulya and Stryer, 1992). The exposure of the myristoyl group targets the protein to membranes where it interacts with target proteins (Ames et al., 1997; Zozulya and Stryer, 1992).

In the myristoyl electrostatic switch, the association between the myristoylated protein and the membrane is disrupted by introduction of negative charges into the positively charged polybasic domain. This reduces the electrostatic interactions with acidic phospholipids and results in the displacement of the myristoylated protein from the membrane (McLaughlin and Aderem, 1995). An example of a protein whose membrane binding is regulated by a myristoyl electrostatic switch is myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS is an acidic protein regulated by Ca^{2+}/calmodulin and protein kinase C that plays important roles in cell shape, motility, secretion, transmembrane transport, and regulation of the cell cycle (McLaughlin et al., 2005). MARCKS is bound to the plasma membrane by a myristoyl moiety and a polybasic domain. When PKC phosphorylates residues within the polybasic domain of the protein, the electrostatic contribution of this region to the membrane binding is greatly reduced (Larsson, 2006). Since the myristoyl moiety is not hydrophobic enough to confer stable membrane association, MARCKS is released from the membrane (McLaughlin et al., 2005; Sundaram et al., 2004; Taniguchi, 1999).

In the myristoyl proteolytic switch, the activity of an endopeptidase induces a conformational change that results in the myristoyl moiety being sequestered inside a hydrophobic pocket within the protein and its consequent dissociation from the membrane. An example of myristoyl proteolytic switch is the HIV-1 Gag precursor, Pr55gag. This protein binds to the plasma membrane via a myristoyl moiety and a polybasic domain. Cleavage of Pr55gag by HIV-1 protease triggers a myristoyl switch that results in formation of the p17MA product, sequestration of the myristoyl moiety, and release of p17MA from the membrane (Resh, 2005).

Since it was first described in 1982 (Aitken et al., 1982), myristoylation was generally considered a co-translational event that occurred while the nascent polypeptide chain was still attached to the ribosome and resulted in a stably modified protein (Resh, 1999). Indeed, the co-purification of NMT associated to the ribosomes supported the assumption (Glover et al., 1997). However in 2000, Zha et al. described for the first time that the pro-apoptotic protein BID underwent post-translational (rather than the classic co-translational) N-myristoylation. This work showed that upon BID cleavage by caspase-8, an internal myristoylation consensus sequence with an amino-terminal glycine residue was exposed on the p15 cleavage product and subsequently myristoylated (Zha et al., 2000). N-myristoylation enables the targeting of a complex of p7 and myristoylated p15 fragments of BID to mitochondria where it enhances cytochrome c release and cell death (Zha et al., 2000).

After the original demonstration of post-translational myristoylation (Zha et al., 2000), two other proteins, actin and gelsolin, were also shown to undergo post-translational myristoylation upon caspase-3 cleavage (Sakurai and Utsumi, 2006; Utsumi et al., 2003). Actin and gelsolin are both involved in cytoskeleton structure and dynamics (Rao and Li, 2004; Silacci et al., 2004). Using a recombinant expression system, Sakurai and Utsumi showed that post-translationally myristoylated FLAG-tagged actin co-localized with mitochondria, like myristoylated p15 Bid (Sakurai and Utsumi, 2006). Interestingly, they also showed that post-translationally N-myristoylated HA-tagged tGelsolin did not localize to mitochondria but appeared cytosolic and had anti-apoptotic activity (Sakurai and Utsumi, 2006).

Finally, studies indicate that many caspase-cleaved proteins expose an amino-terminal glycine residue (Maurer-Stroh et al., 2002; Nicholson, 1999), suggesting that other post-translationally myristoylated proteins may exist and that post-translational myristoylation might be a cellular mechanism used to regulate cell death. Exploring this new role for myristoylation in temporal and spatial regulation of signalling events constitutes a key area for future investigation.

1.1.4.3 Other types of Fatty Acylation

Fatty acylation of proteins has been shown to be more diverse than the addition of palmitoyl and myristoyl moieties to cysteine or glycine residues. Other types of "unusual" fatty acylation include serine octanoylation, lysine

myristoylation and lysine palmitoylation. In each case the enzymes responsible for the acylation reactions in eukaryotes are unknown.

Ghrelin is a peptide produced predominantly in the stomach that stimulates feeding and growth hormone (GH) secretion. Acylation by octanoate through an oxyester bond to a serine residue is indispensable for the binding of ghrelin to the GH secretagogue type 1a receptor (GHS-R1a), the functionally active form of the GHS-R (Kojima et al., 1999; Toshinai et al., 2006).

Although proven to be myristoylated, a few proteins including the insulin receptor, the μ immunoglobulin heavy chain, tumor necrosis factor α, and the interleukin 1α and 1β (IL-1α and IL-1β) precursors, lack glycine residues correctly positioned for N-myristoylation. Interestingly, myristoylation of these proteins was shown to occur via an alternative mechanism in which the free ε-amino groups of internal lysine residues form the characteristic amide bonds (Bursten et al., 1988; Hedo et al., 1987; Pillai and Baltimore, 1987; Stevenson et al., 1993; Stevenson et al., 1992).

Interestingly, certain toxins from spider venom as well as myelin lipophilin (also known as proteolipid apoprotein or Folch-protein) were shown to be modified by a palmitoyl moiety via an oxyester linkage on a threonine residue (O-palmitoyl threonine) at or near their carboxy-terminus (Branton et al., 1993; Stoffel et al., 1983).

Finally, in the Gram negative bacteria *Escherichia coli*, the pore-forming protein hemolysin (HlyA) has been shown to require the post-translational acylation of internal lysine residues for proper activity. The inactive protoxin pro-

HlyA is activated intracellularly by amide linkage of fatty acids to two internal lysine residues 126 amino acids apart in a reaction directed by the co-synthesized HlyC protein with acyl carrier protein as the fatty acid donor (Stanley et al., 1998).

1.2 The role of protein fatty acylation in specific proteins

Clearly, when attached to proteins myristate and palmitate have different functions as illustrated above and removal of these acyl moieties severely impairs the normal function of the modified proteins. The different types of fatty acylated proteins currently known suggest that the modification of selected proteins with myristic and/or palmitic moieties acts as a molecular switch specifically and selectively altering their physical and biological properties. Identification and understanding of the function of these various fatty acylated proteins is important to our understanding of the dynamic and vital role of this modification in cellular biology and constitute the main priority of our laboratory. Herein we will characterize the role of palmitoylation in apolipoprotein B secretion and the role of post-translational myristoylation of p21-activated protein kinase 2 (PAK2) during apoptosis. Therefore, an introduction to the biology of these two proteins is presented below.

1.2.1 Apolipoprotein B

Apolipoprotein B is a large amphipathic protein (Segrest et al., 2001), which exists in two forms: Apolipoprotein B 100 (apoB-100) and Apolipoprotein B 48 (apoB-48). In humans, the gene for apoB is located at the chromosome 2 (Chen

et al., 1987). The coding region of the apoB gene has more than 43 kb and encompasses 28 introns and 29 exons (Wang et al., 2003). The gene can code for two different isoforms of the apoB protein, namely apoB-100 and apoB-48. The apoB-100 gene product is composed of 4536 amino acids and constitutes the largest monomeric polypeptide synthesised by the human body (Wang et al., 2003). The apoB-48 gene product is produced via a post-transcriptional site specific mRNA editing process during which a deamination of a cytidine to a uridine in nucleotide 6666, converts a glutamine codon (CAA) to a stop codon (UAA) at approximately 48% of the full-length coding sequence (Innerarity et al., 1996; Wang et al., 2003), hence the name apoB-48 for the resulting truncated protein. This apoB mRNA editing process has been shown to occur in the small intestine of all mammals and in the liver of rats, mice, dogs, and horses (Greeve et al., 1993). ApoB-100 and apoB-48 play a key role in the blood transport and metabolism of hydrophobic lipids such as triacylglycerols and cholesterol (Davis, 1999; Davis, 1996; Havel, 1995; Hussain et al., 2005). In humans, apoB-100 is synthesized exclusively in the liver and it is an essential structural component of very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low-density lipoproteins (LDL). Human apoB-48 is produced by the small intestine and is required for the production of chylomicrons (CM) and their remnants (Hussain et al., 2005; Olofsson and Boren, 2005).

Both isoforms of apoB have a complex structure and the main difference between them is the presence of a LDL receptor-binding domain at the carboxy-terminal half of apoB-100. This domain is absent in the shorter apoB-48 isoform

(Hussain et al., 2005). The lack of the LDL receptor-binding domain determines a differential influence for both proteins in the catabolism of lipoproteins (Hussain et al., 2005; Kendrick et al., 2001).

Structurally, apoB-100 is organized in five well defined domains. It consists of one globular amino-terminal domain, two amphipathic β-sheet domains and two amphipathic α-helix domains (Gursky, 2005; Segrest et al., 2001). The region encompassing the first 1000 amino acids of apoB-100 is homologous to the lipid-binding pocket of the lamprey protein vitellogenin. This amino-acid sequence is of utmost importance for the formation of VLDL, since it is the region of the protein that interacts with the microsomal triglyceride transfer protein (MTP). MTP is the protein responsible for the transfer of lipids to apoB during the formation of lipoproteins (Dashti et al., 2002). The amphipathic β-sheet domains consist of antiparallel β-sheets that form very strong lipid-binding structures (Segrest et al., 2001). It is believed that these domains are responsible for preventing apoB-100 from transferring between different lipoprotein particles and keeping it bound to the original particle on which it was secreted into the plasma (Olofsson and Boren, 2005). Alternated between the two amphipathic β-sheet domains, there is an amphipathic α-helix domain with the remaining one present near the carboxy-terminus of the protein (Segrest et al., 2001). Although the three-dimensional structure of apoB-100 is not known in detail, the overall organization of the protein on the LDL particle has been deduced based on the immuno-localization of epitopes, electron microscopy and site-directed mutagenesis (Chatterton et al., 1995). These studies have

generated a model where apoB-100 has an elongated structure encompassing the entire particle. The model predicts that the carboxy-terminus of the protein folds back over the preceding structure, crossing it at amino acid 3500. At this position, the arginine residue 3500 binds the tryptophan residue 4396 and prevents the carboxy-terminus from sliding over the binding site for the LDL receptor (Chatterton et al., 1995; Gursky, 2005; Olofsson and Boren, 2005). Interestingly, mutations on these amino acids have been shown to result in a pathological condition known as familial ligand defective apoB-100 or FDB (Whitfield et al., 2004). FDB is a disorder of LDL metabolism characterized by hypercholesterolemia and premature atherosclerosis (Innerarity et al., 1987). Several mutations in the *APOB* gene affecting the binding affinity for the LDL receptor have been identified. The most common, R3500Q, affects approximately 1 in 500 individuals of European descent and involves the substitution of a glutamine for an arginine residue at position 3500 (Innerarity et al., 1990; Soria et al., 1989). Finally, the carboxy-terminal portion of the protein appears to interact with the amino-terminus thus forming a ring around the particle (Gursky, 2005).

Interestingly, all apoB-containing lipoprotein particles such as VLDL, IDL and LDL are considered to be atherogenic since their plasma concentrations have been shown to correlate positively with the trend to develop atherosclerosis (Havel, 1995). Atherosclerosis is a progressive disease and the principal cause of cardiovascular diseases (CVD) in the western world. CVD are the leading cause of death and disability in industrialized societies (Cheng et al.,

2002; Hajjar, 1995). Each year, about 50% of the deaths in North America are caused directly by atherosclerosis or other related CVD (http://www.who.int/topics/cardiovascular_diseases/en/, 2006). During the progression of atherosclerosis, cholesterol (along with lipids, cellular debris and fibrin) accumulates in the walls of arteries forming plaques that obstruct the flow of blood. This process eventually leads to an increased arterial blood pressure, cardiac hypertrophy and heart failure (Fliegel and Karmazyn, 2004; Govindarajan et al., 2005; Nieminen and Harjola, 2005). At the circulatory level, the eventual rupture of the plaque and the consequent thrombosis may lead to sudden arterial blockage and cause a heart attack (Benetis, 2005; Govindarajan et al., 2005; Ho et al., 1993).

Since apoB is required for the assembly of many atherogenic lipoproteins, it is important to understand its biosynthetic process in order to design strategies that will allow controlling the circulating concentration of apoB, thereby managing the levels of cholesterol and triglycerides in the blood.

Like most secreted proteins, apoB biogenesis starts in the endoplasmic reticulum (ER). Unlike other secreted proteins, it needs the association of lipids to form a mature lipoprotein particle. A model for the assembly of apoB-containing lipoprotein particles has been proposed that involves a two step process (Fig. 1.4) (Alexander et al., 1976; Boren et al., 1994; Hamilton, 1995).

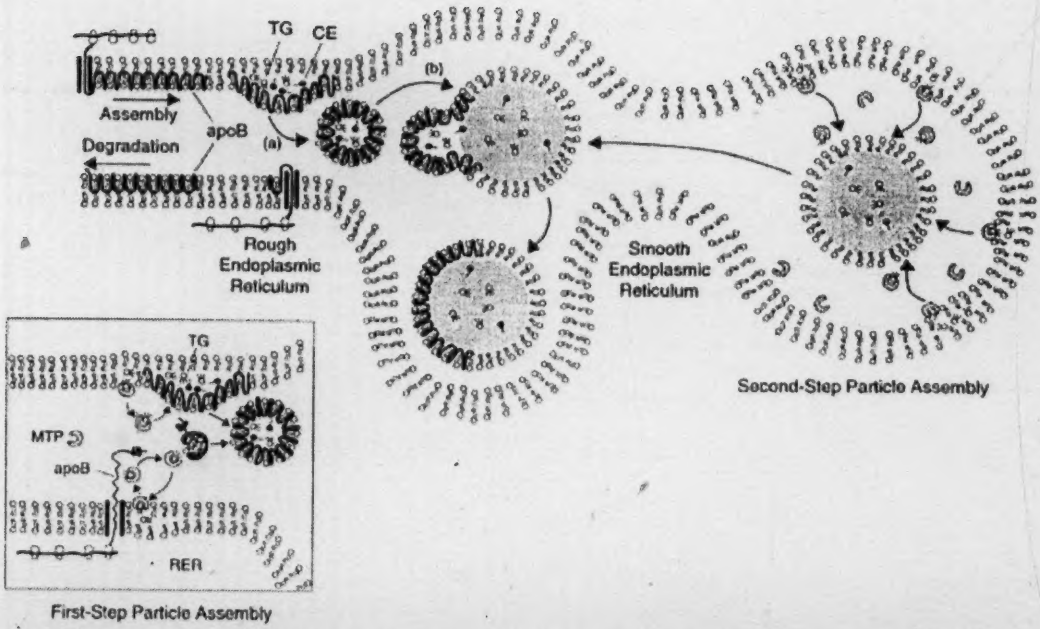


Figure 1.4. **A two-step model for the assembly of apoB-containing lipoprotein particles.** In the first step of the assembly process, apoB is translated and translocated into the ER where associated with the inner leaflet of the membrane. If neutral lipids are available, an apoB-containing lipoprotein precursor is formed in a process dependent on the activity of the microsomal triglyceride transfer protein (MTP). If during this assembly step the availability of neutral lipids is low, apoB is retro-translocated to the cytosol, ubiquitinated and degraded by proteosomes. During the second assembly step, the apoB-containing lipoprotein precursor fuses with a lipid droplet at the smooth termini of the ER in an MTP independent fashion. Once the fusion is complete, the nascent lipoprotein particle reaches an ER exit site and continues through the secretory pathway. TG, triacylglycerol; CE, cholesteroyl ester; RER, rough endoplasmic reticulum. Adapted from Hamilton *et al.* (Hamilton et al., 1998).

In the first step of this model, the assembly of an apoB-containing lipoprotein particle starts in the rough ER when the nascent apoB polypeptide enters the secretory pathway during its translation (Olofsson and Boren, 2005). A pre-VLDL lipoprotein is formed when the growing apoB polypeptide is complexed with small amounts of triacylglycerol and cholesteroyl esters, in a process that is dependent on the activity of MTP, an ER resident protein (Berriot-Varoqueaux et al., 2000). MTP adds lipids to the growing apoB molecule allowing it to fold on a hydrophobic core (Olofsson et al., 1999; Olofsson and Boren, 2005). This results in the formation of a pre-VLDL lipoprotein particle, which has a small size (Boren J et al., 1992) and is retained in the cell, in part because of interaction with chaperone proteins (Stillemark-Billton et al., 2005). Association of calnexin (CLX) and calreticulin (CRT) coincides with the assembly of apoB into lipoprotein particles thus suggesting an overlap in the time of association of newly synthesized apoB with MTP and CLX/CRT (Tatu and Helenius, 1999). Therefore, it is possible that CLX and CRT binding may facilitate MTP dependent transfer of triglycerides onto nascent apoB. Additional lipidation of the pre-VLDL particle by MTP produces an immature, triglyceride-poor VLDL (Stillemark-Billton et al., 2005). The additional lipidation is necessary for the secretion of apoB and its extent is directly related to the length of the protein. Extensive structure/function studies performed on carboxy-terminally truncated forms of apoB have established that while apoB-18 binds little or no lipids, the size of lipoprotein particles increases and their density decreases almost in a linear fashion from apoB-25 to apoB-80 (Spring

et al., 1992; Yao et al., 1991). Studies have shown that the regulation of apoB secretion is post-translational and depends on the availability of neutral lipids (Borchardt and Davis, 1987; Bostrom et al., 1988). If during the assembling process these neutral lipids are not available or MTP activity is impaired, the newly synthesized apoB protein associates with the inner leaflet of the ER membrane where it potentially waits to be lipidated (Mitchell et al., 1998). If lipidation does not take place, apoB is retrotranslocated to the cytosol and eventually degraded by the proteasome following ubiquitination (Fisher et al., 2001).

In the second step of the assembly model, the apoB-containing VLDL precursor fuses with a neutral lipid droplet to yield a mature VLDL particle and moves along the secretory route (Hamilton et al., 1998). Borchardt and Davis demonstrated that the exit from the ER is the rate-limiting step in the apoB secretion process (Borchardt and Davis, 1987). For many years it was a matter of debate whether the lipid loading process occurs exclusively at the smooth ends of the rough ER (Alexander et al., 1976; Rusinol et al., 1993) or if it also continues in the Golgi apparatus (Bamberger and Lane, 1990; Bostrom et al., 1988; Cartwright and Higgins, 1995; Higgins, 1988; Swift et al., 2001). Recently, Asp et al. demonstrated that the formation of VLDL was dependent on the GTPase ARF 1 (Asp et al., 2005). This group showed that transient expression of ARF-1(T31N), a GTP-binding deficient mutant, significantly inhibited apoB-100 VLDL production without influencing the biosynthesis of apoB-100 LDL or total apoB production. Interestingly, this inhibition did not alter total protein

production, biosynthesis of transferrin, phosphatidylcholine or triglycerides either (Asp et al., 2005). Given that ARF 1 is required in the anterograde transport from ERGIC to cis-Golgi, it appears that the lipid loading process of VLDL could take place in the early Golgi apparatus (Asp et al., 2005).

In addition to the availability of lipids, studies have shown that post-translational modifications of apoB, including disulfide bond formation, glycosylation, and palmitoylation have an impact on the assembly and secretion dynamics of lipoproteins containing apoB (Davis, 1999; Davis, 1996; Havel, 1995). Most of these studies were done *in vitro* using carboxy-terminally truncated apoB forms as a model to test the function and effects of such modifications. These truncated apoB proteins displayed lower secretion efficiency, enhanced intracellular degradation, and impaired ability to assemble into lipid-rich lipoproteins (Huang and Shelness, 1997; Tran et al., 1998). The fatty acylation of human apoB was originally demonstrated by Hoeg et al. (Hoeg et al., 1988) and the first palmitoylation site was identified in our laboratory by Zhao et al. (Zhao et al., 2000). Using transfected rat hepatoma McA-RH7777 cells, Vukmirica et al. also demonstrated that apoB-48 is palmitoylated on additional cysteine residues (Vukmirica et al., 2002). ApoB-100 contains 25 cysteine residues, sixteen of which are involved in the formation of eight disulfide bonds (Yang et al., 1990). One of such residues, located at the carboxy-terminus of the protein, is involved in covalent linkage with apolipoprotein (a) (Gabel et al., 1994), and the remaining eight cysteine residues are susceptible to modification by palmitoylation. Although, it has been

demonstrated that apoB100 secreted from HepG2 cells was able to incorporate a [^{14}C]palmitate via a hydroxylamine-sensitive thioester bond (Hoeg et al., 1988; Huang et al., 1988), the total number of cysteine residues that are modified by palmitoyl moieties in the protein is not known (Huang et al., 1988). However, studies performed by Zhao *et al* in our laboratory, unequivocally identified cysteine residue 1085 in human apoB-29 as palmitoylated. The mechanism by which the secreted apoB protein gets palmitoylated is currently unknown (Zhao et al., 2000). However, as discussed in the previous section, members of the MBOAT family of enzymes could be involved in this process. The assembly of apoB-containing lipoprotein particles appears to be initiated when the apoB polypeptide is translocated into the lumen of the ER and associates with the inner leaflet of its membrane (Olofsson and Boren, 2005). Therefore, association of apoB with membrane represents an important step in the VLDL assembly dynamics. It is possible that palmitoylation of cysteine residues within the apoB protein might contribute to the stabilization of the protein in the membrane of the ER. Interestingly, Zhao *et al*, found that palmitoylation at cysteine residue 1085 was likely involved in the assembly of the hydrophobic lipid core of the lipoprotein particle since secreted lipoprotein particles containing non-palmitoylated (Cys1085Ser) apoB-29 contained 5 times less neutral lipids than those containing palmitoylated apoB-29 while phospholipid levels present in the monolayer shell of both types lipoprotein particles remained unaffected (Zhao et al., 2000). Furthermore, under steady state conditions, most of the palmitoylated apoB-29 appeared to be found in large vesicular structures

containing endogenous apoB-100 which corresponded to a sub-compartment of the ER while non-palmitoylated (Cys1085Ser) apoB-29 appeared to be concentrated in a dense perinuclear area corresponding to the Golgi compartment (Zhao et al., 2000). Studies performed by Vukmirica et al. utilizing McArdle-RH7777 cells transiently expressing apoB-48 and a mutated version where all 4 potentially palmitoylatable free cysteine residues of the protein were substituted by alanine, showed that mutant apoB-48 presented a retarded movement toward the distal Golgi and an increased association (>2-fold) with the membranes of the secretory compartments (Vukmirica et al., 2003). In the same work these authors also observed a 15-20% decrease in the secretion efficiency of the mutant protein as compared with that of wild type apoB48 (Vukmirica et al., 2003).

These results represent the first exciting evidence of the functional role of palmitoylation in apoB assembly and open many questions regarding the involvement of this modification during the assembly and secretion of lipoprotein particles. Moreover, our work on the characterization of the single palmitoylated cysteine residue 1085 on apoB-29 adds further precision on the role of palmitoylation in apoB biogenesis (Vilas and Berthiaume, 2004).

1.2.2 p21 activated kinase 2 (PAK2) and programmed cell death

Our work identified PAK2 as the second post-translationally myristoylated protein at the endogenous level during programmed cell death (Vilas et al.,

2006). The sections below will introduce roles and functions of PAK2 and give an overview of programmed cell death.

1.2.2.1 Biology of PAK2

PAK2 is a member of the p21-activated protein kinase family. The mammalian PAK family consists of six members that are differentially expressed in mammalian tissues (Daniels and Bokoch, 1999) and can be divided into two subfamilies according to sequence homology (Bokoch, 2003). The first subfamily consists of PAK1, PAK2, and PAK3 and the second subfamily consists of the more recently identified PAK4, PAK5, and PAK6 (Jakobi et al., 2003). Although the latter have also been termed PAKs, PAK4–6 differ significantly in their structural organization and regulation from PAK1–3 (Bokoch, 2003) and are out of the scope of this work.

PAK1–3 are serine/threonine protein kinases whose catalytic domains have a high degree of homology with Ste20, a protein kinase from the budding yeast *Saccharomyces cerevisiae* (Dan et al., 2001). Structurally, PAK1-3 are organized in an amino-terminal regulatory domain that contains several protein-protein interaction motifs and a highly conserved carboxy-terminal catalytic domain (Bokoch, 2003). PAKs 1-3 exist as cytosolic homodimers, with the amino-terminal regulatory domain of one molecule bound and inhibiting the carboxy-terminal kinase domain of the other (Bokoch, 2003; Knaus et al., 1995). The catalytic activity of PAK1-3 is regulated by the binding of the GTP-bound form of Rac and CDC42 to a highly conserved motif in the amino-terminal

regulatory domain of the proteins, known as the p21-binding domain (PBD) or Cdc42/Rac interactive binding (CRIB) domain (Bokoch, 2003; Knaus et al., 1995). This binding is thought to induce a conformational change in PAKs that relieves the inhibition of the catalytic carboxy-terminal domain and promotes cross-phosphorylation at several sites thus enabling the kinases to phosphorylate their specific substrates.

PAKs are activated in response to extra cellular signals and regulate cell shape and motility as well as cell survival and programmed cell death. Of the six members of the PAK family, PAK2 is the only one that is ubiquitously expressed (Bokoch, 2003; Jakobi et al., 2003; Jakobi et al., 2001; Knaus et al., 1995).

Stimulation of PAK2 kinase activity occurs in response to various forms of moderate stress such as serum starvation, DNA damage, ionizing radiation, heat shock, and hyperosmolarity (Bokoch, 2003). Upon induction of apoptosis, PAK2 is constitutively activated via cleavage by caspase-3 and functions to promote apoptosis (Jakobi et al., 2003; Rudel et al., 1998; Vilas et al., 2006). Caspase-3 cleavage of PAK2 removes most of the amino-terminal regulatory domain and generates a constitutively active C-t-PAK2, consisting of a 34 kDa carboxy-terminal fragment that contains the entire catalytic domain (Rudel and Bokoch, 1997). Interestingly, PAK2 appears to be the only member of the PAK family that is proteolytically processed by caspases (Bokoch, 2003; Rudel and Bokoch, 1997).

Expression of recombinant C-t-PAK2 was shown to induce morphological changes characteristic of apoptotic cell death in a variety of cell lines and

increased cell death in Hela, CHO, Jurkat and COS-7 cells (Jakobi et al., 2003; Lee et al., 1997; Rudel and Bokoch, 1997; Rudel et al., 1998; Vilas et al., 2006). Activated PAK2 has also been shown to be involved in the down regulation of translation initiation during apoptosis (Orton et al., 2004). This suggests that this caspase-dependent mechanism of PAK2 regulation might be an important contributor to downstream signalling events leading to the morphological and physiological changes characteristic of apoptotic cells.

1.2.2.2 Programmed cell death

Programmed cell death or apoptosis, is a fundamental process in the development of multicellular organisms. Apoptosis enables an organism to eliminate unwanted or defective cells through an organized process of cellular disintegration that has the advantage of not inducing an inflammatory response. Unlike necrosis, which is an accidental and passive process, the launching of the apoptotic program involves the activation of energy-requiring intracellular machinery. This specialized machinery is tightly regulated and conserved throughout evolution (Yuan, 1996). Apoptosis affects single cells asynchronously, typically in the absence of damage for its surrounding neighbours (Wyllie, 1997).

The apoptotic process plays a key role in the control of normal embryonic morphogenesis and development of the immune, endocrine and nervous system as well as in homeostasis of adult organs and tissues (Green, 2005). For example, during fetal and postnatal development, apoptosis contributes to

normal structural maturation of the lung (Scavo et al., 1998; Schittny et al., 1998). Apoptosis also eliminates cells exposing the organism to danger such as virally infected cells or cells with damaged DNA (Vermeulen et al., 2005). Improper regulation of apoptosis has been associated with disorders such as cancer (Vermeulen et al., 2005), viral infection, autoimmune diseases, neurodegenerative disorders (Mahoney and Rosen, 2005), stroke, anaemia and AIDS (Fadeel and Orrenius, 2005).

The term apoptosis was first used by Kerr *et al* in 1972 to describe the morphological pattern of cell death observed during embryonic development, normal cell turnover in healthy adult tissue, and growth factor withdrawal (Kerr et al., 1972). During the apoptotic process, plasma membrane integrity is maintained but it undergoes composition changes such as exposure of phosphatidylserine. Other morphological characteristics of cells in a more advanced apoptotic stage include cellular shrinkage, membrane blebbing, nuclear chromatin condensation and fragmentation. Eventually, the cell breaks into membrane-surrounded fragments (apoptotic bodies), which are engulfed *in vivo* by "professional" phagocytes (macrophages and dendritic cells). In cell cultures, apoptotic bodies will lose the integrity of the plasma membrane during the late stages of apoptosis, followed by complete cell disintegration, in a process called secondary necrosis (Wyllie et al., 1980).

The core of the apoptotic process is a proteolytic system involving a group of proteases called cysteinyl-aspartyl-proteases or caspases, which are involved in the initiation and execution of this process. All caspases have similar amino

acid sequence substrate specificity and structure. They are organized in three domains: an amino-terminal pro-domain, a large subunit and a small subunit (Vermeulen et al., 2005).

Caspases are synthesized as inactive proenzymes but they can be rapidly activated by auto-proteolytic cleavage or cleavage by other caspases at specific tetrapeptide sequences containing an aspartic acid residue (Vermeulen et al., 2005). So far, 14 members of the caspase family have been identified. However, only a little over half of the family members (caspase-2, -3, -6, -7, -8, -9, -10 and -12) have been shown to be part of the apoptotic process (Vermeulen et al., 2005). The remaining caspases are involved in processing pro-inflammatory cytokines and in mediating inflammatory responses (Thornberry et al., 1992). Depending on the length of their pro-domain, the caspases involved in apoptosis can be further divided into two groups: long pro-domain initiator caspases (caspase-2, -8, -9, and -10) and short pro-domain effector caspases (caspase-3, -6, and -7) (Yan and Shi, 2005). Upon triggering of apoptosis, initiator caspases act as upstream signal transducers and proteolytically activate downstream effector caspases (Wang et al., 2005). The long amino-terminal pro-domains of initiator caspases contain protein/protein interaction motifs. While initiator caspases -8 and -10 contain a death effector domain (DED) in their pro-domain that is involved in interactions with adaptor proteins, caspases -2 and -9 present a caspase recruitment domain (CARD), important for binding of adaptor molecules and activation of effector caspases, in their pro-domain (Fadeel and Orrenius, 2005). Activated effector caspases cleave several types

of substrates whose activation (or inactivation) ultimately result in the cellular and morphological alterations characteristic of apoptosis (Cohen, 1997).

In general, the functions of initiator caspases can be summarized as to: (1) arrest the cell cycle and inactivate DNA repair; (2) inactivate the inhibitor of apoptosis (XIAP); and (3) dismantle the cellular cytoskeleton (Fink and Cookson, 2005; Wang et al., 2005). Although caspase cleavage of different proteins is a critical event for apoptotic execution, many key caspase substrates are likely to remain to be discovered. However, the identity and function of many important caspase targets are well known and understood. One of the first proteins cleaved by effector caspases is poly-(ADP-ribose) polymerase (PARP). PARP is a nuclear protein implicated in DNA repair and is targeted for specific caspase cleavage in order to inactivate this mechanism (Duriez and Shah, 1997). DNA-PK, a serine/threonine protein kinase that is critical to a pathway of double strand DNA damage repair termed non-homologous end joining (NHEJ), is also cleaved by caspases in order to dismantle the DNA repair system. Caspase cleavage and inactivation of ICAD (inhibitor of caspase-activated DNAse) allows CAD [also known as DNA fragmentation factor (DFF)] to translocate to the nucleus where it is responsible for internucleosomal DNA cleavage and generation of oligonucleosomal DNA fragments (Liu et al., 1997; Sakahira et al., 1998).

In order to arrest the cell cycle, caspases cleave key regulators such as retinoblastoma protein (pRb), important transcription factors like NF-κB and cell signalling proteins [e.g. Raf, protein kinase B (PKB)] (Hengartner, 2000). The

cell survival factors Bcl-2 and Bcl-Xl are also cleaved during apoptosis, as are the pro-apoptotic proteins Bid and Bax (Fadeel and Orrenius, 2005). Finally, caspase cleavage of lamins results in nuclear shrinkage and cleavage of many important cytoskeletal proteins like fodrin, p21 activated kinase 2 (PAK2), gelsolin and actin leads to cell shrinkage and membrane blebbing (Bokoch, 2003; Mollinedo and Gajate, 2003; Orth et al., 1996; Utsumi et al., 2003).

Two distinct but interconnected apoptotic pathways regulate caspase activation and cellular death. The extrinsic or receptor mediated pathway involves cross-linking of death receptors following extra cellular stimuli and the intrinsic pathway, where the apoptogenic factors are released from the mitochondria following intracellular signals (Fig. 1.5) (Hengartner, 2000; Leist and Jaattela, 2001). In addition, two other apoptotic pathways are emerging: endoplasmic reticulum stress-induced apoptosis and caspase-independent apoptosis.

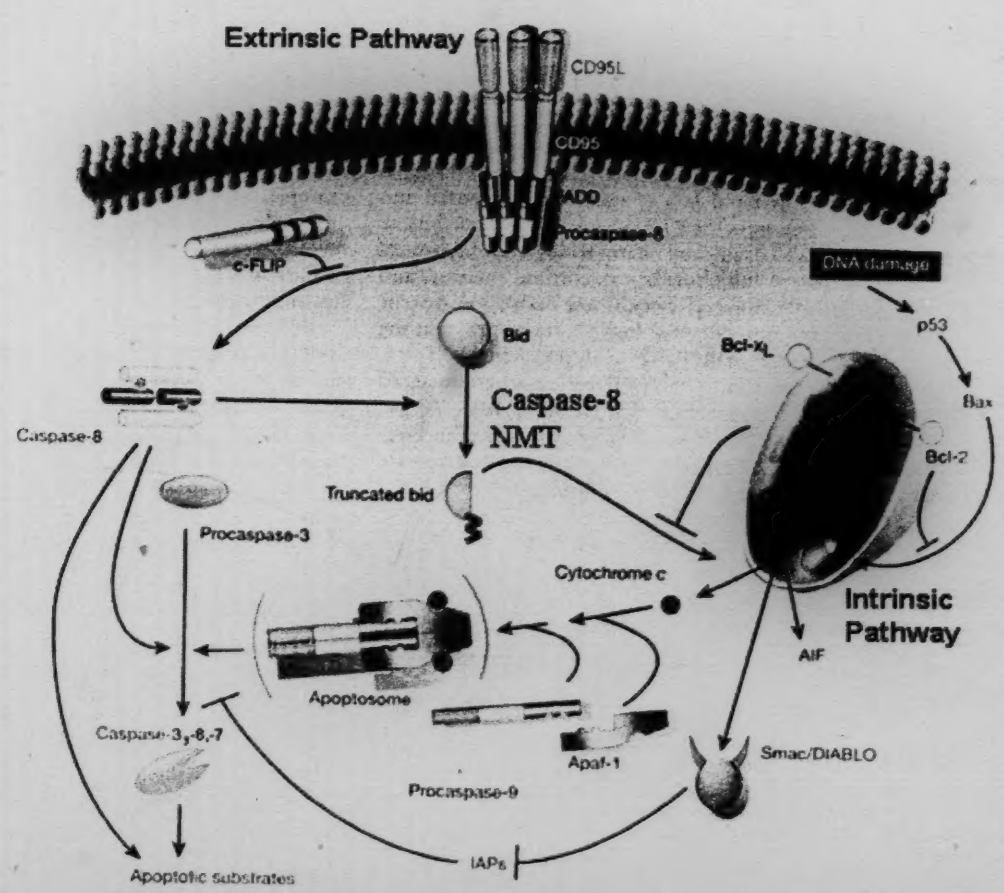


Figure 1.5. **The two major apoptotic pathways in mammalian cells.** The intrinsic or mitochondrial apoptotic pathway is activated by several stress stimuli such as starvation or DNA damage. These stresses lead to loss of mitochondrial membrane potential and the release of several mitochondrial proteins including cytochrome c. Release of cytochrome c from the mitochondria causes the activation of the apoptotic protease activating factor-1(Apaf-1) and recruitment of procaspase-9. Following activation, the apoptosome-associated caspase-9 will in turn activate downstream caspases like caspase-3, caspase-6 and caspase-7. The receptor-mediated pathway is activated by the binding of specific ligands [e.g. tumour necrosis factor (TNF) or Fas/CD95 ligand]. Binding of Fas ligand (FasL) to Fas induces the trimerization of the receptor, which leads to the recruitment of the Fas-associated death domain protein (FADD) and activation of caspase-8. Active caspase-8 then cleaves the pro-apoptotic protein Bid and activates downstream effector caspases by cleaving pro-caspase-3. Truncated Bid is then myristoylated and targeted to the mitochondria where it activates the intrinsic pathway. Adapted from **Hengartner (Hengartner, 2000).**

1.2.2.2.1 Receptor-mediated apoptotic pathway

The receptor-mediated pathway is activated by the binding of specific ligands [*e.g.* tumour necrosis factor (TNF) or Fas/CD95 ligand] to a certain type of plasma membrane receptors, known as death receptors, which trigger the apoptosis process (Kim, 2002; Vermeulen et al., 2005). Such receptors belong to the tumour necrosis factor (TNF)-receptor superfamily. This family includes Fas (Apo-1 or CD95), TNF-receptor-1 (TNF-R1), death receptor-3 [DR3 or TNF-receptor-related apoptosis-mediating protein (TRAMP) or Apo-3], TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1 or DR4), TRAIL-R2 (DR5 or Apo-2) and DR6 (Fadeel and Orrenius, 2005). The best studied of all the above mentioned death receptor is Fas.

Binding of Fas ligand (FasL) to Fas induces the trimerization of the receptor, which leads to the recruitment of the Fas-associated death domain protein (FADD) (Kim, 2002). FADD contains two different domains called the "death effector domain" (DED) and the "death domain" (DD) located at its amino- and carboxy-terminus, respectively. The Fas receptor contains a death domain (DD) in the cytoplasmic tail that binds FADD via its carboxy-terminal death domain (FADD-DD) forming a death receptor-induced signalling complex (DISC) (Kim, 2002; Vermeulen et al., 2005). Then the amino-terminal death effector domain of FADD (FADD-DED) recruits the DED-containing procaspases -8 or -10 into the DISC (Medema et al., 1997). Upon recruitment, the DED-containing procaspases are proteolytically activated to their

enzymatically active form, which in turn activate downstream effector caspases (Thornberry and Lazebnik, 1998).

Activation of caspases through other death receptors occurs in a similar way. For example, binding of TNF-α to TNF-R1 leads to its trimerization and recruitment of the TNF-R-associated death domain protein (TRADD). The death domain of TRADD binds the death domain of FADD, which in turn recruits pro-caspase-8 and activates it. Active caspase-8 then activates downstream effector caspases by cleaving pro-caspase-3 (Stennicke et al., 1998). Interestingly, TNF-α binding to TNF-R alone seldom induces apoptosis. This is because the binding of the adaptor molecule TNF receptor-associated factor-2 (TRAF2) to TRADD recruits cellular inhibitor of apoptosis (c-IAP)-1 and c-IAP-2, two anti-apoptotic proteins (Vermeulen et al., 2005). The binding of the receptor interaction protein (RIP), the third protein able to interact with TRADD, leads to activation of the transcription factor NF-κB, resulting in transcription of anti-apoptotic genes and promoting cell survival (Ashkenazi and Dixit, 1998; Nagata, 1997; Wang et al., 2005). Alternatively, the interactions between RIP and TRADD can trigger the onset of apoptosis by recruiting caspase-2 through the adaptor molecule RIP-associated ICH-1/CED3 homologous protein with DD (RAIDD) (Fadeel and Orrenius, 2005; Vermeulen et al., 2005). Other death receptor (DR)-activating ligands include lymphotoxin, Apo3-ligand and Apo2-ligand. They recognize and bind with their respective receptors (TNF-R1, DR3, DR4 and DR5) and adaptor molecules. In addition to death receptors, decoy receptors (DcR1, DcR2, DcR3, osteoprotegerin) have been identified (Wang et

al., 2005). These receptors compete with the death receptors for ligand binding, but they do not transduce apoptotic signals (Wang et al., 2005).

1.2.2.2.2 Intrinsic apoptotic pathway

The intrinsic or mitochondrial pathway is activated by a variety of extra cellular cues and intracellular insults such as DNA damage, serum withdrawal and heat shock. These stresses lead to loss of mitochondrial membrane potential ($\Delta\Psi m$) and the release of several proteins, including cytochrome c. Release of cytochrome c from the mitochondria causes the activation of the apoptotic protease activating factor-1 (Apaf-1) (Wang et al., 2005). The CARD domain of Apaf-1 binds with the CARD domain of procaspase-9 in a cytochrome c and ATP dependent manner forming the mitochondrial DISC, or apoptosome. Following activation, the apoptosome-associated caspase-9 will in turn activate downstream caspases like caspase-3, caspase-6 and caspase-7 (Vermeulen et al., 2005).

The way in which the mitochondria release cytochrome c upon an apoptotic insult is unclear. It is believed that the release occurs via the mitochondrial permeability transition pore (PTP), a structure that is formed at the contact points between the inner and outer mitochondrial membranes. The PTP is mainly composed of a voltage-dependent anion channel (VDAC) that faces the cytosol and an adenine translocator (ANT) in the matrix side of the contact point. Other proteins are associated to the cytoplasmic face of the complex: hexokinase II (HKII), mitochondrial creatine kinase (mtCK), cyclophilin D (Cyp-

D) and peripheral benzodiazepine receptor (PBR) and are involved in its regulation (Baines et al., 2005; Festjens et al., 2004). Many intracellular cues regulate the opening of the PTP complex. The most studied of these effectors are: cations like Ca^{2+}, Mg^{2+}, H^{+}, local changes in the ADP/ATP ratio, and changes in $\Delta_{\Psi m}$ as well as in the Bcl-2 complex (Halestrap, 2006).

In the last years, members of the Bcl-2 protein family have emerged as important regulators of the PTP complex opening. All members of the Bcl-2 family share characteristic domains of homology called the Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4) and are divided in two groups: anti-apoptotic and pro-apoptotic. The anti-apoptotic group of Bcl-2 family of proteins is composed of: Bcl-2, Bcl-W, Boo/DIVA, Mcl-1, Bfl-1/A1, Bcl-Xl and Bcl-B proteins. The pro-apoptotic group of this protein family is further divided into two sub-groups based on whether they contain only one BH3 domain: Bid, PUMA, Spike, Bik/Nbk, Bak, Bad, Nix, Bim, BNIP3, Bmf, Noxa and Harakiri; or more than two different BH domains: Bax, Bcl-Xs, Bfk, Bok/Mtd, Bcl-G_L (Festjens et al., 2004; Vermeulen et al., 2005). The different family members can homo- or hetero-dimerize on the outer mitochondrial membrane, and their relative ratios will determine the susceptibility of cells to the apoptotic stimuli (Festjens et al., 2004).

Although it has been shown that the different composition of the Bcl-2 family proteins polymers can modulate the permeabilization of the mitochondrial membranes and regulate release of cytochrome c (Halestrap, 2006), the mechanism by which the Bcl-2 family members regulate apoptosis has not been

completely determined. Bcl-2 family proteins have also been reported to be involved in the release of other mitochondrial proteins other than cytochrome c that contribute to the homeostatic shut down of the cell. These proteins are: apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI (Smac/Diablo), Omi/HtrA2 and endonuclease G (Vermeulen et al., 2005). After their release from the mitochondria, Smac/Diablo and Omi/HtrA2 interact with proteins called inhibitors of apoptosis (IAPs) such as X-linked inhibitor of apoptosis (XIAP) preventing their activity (van Loo et al., 2002) and endonuclease G translocates to the nucleus and induces caspase-independent DNA fragmentation (Li et al., 2001).

As mentioned earlier, both the receptor mediated and the mitochondrial pathways are tightly interconnected. A molecular link between them is found at the level of caspase-8 cleavage of cytosolic Bid (Li et al., 1998; Luo et al., 1998). Following Fas activation, caspase-8 cleaved Bid [truncated Bid (tBid)] translocates from the cytosol to mitochondria and activates the mitochondrial-dependent apoptotic pathway (Zha et al., 2000). Interestingly, Bid was shown to be post-translationally-myristoylated upon caspase-8 cleavage, and myristoylation enhanced Bid-induced release of cytochrome c and cell death (Zha et al., 2000).

1.2.2.2.3 Endoplasmic reticulum stress-induced apoptotic pathway

Recently, the ER has emerged as another center for regulation of cell death (Ferri and Kroemer, 2001). Prolonged ER stress can be responsible for

the activation of apoptosis through both mitochondrial dependent and independent pathways (Groenendyk and Michalak, 2005). Caspase-12, which is ubiquitously expressed and localized to the ER membrane as an inactive proenzyme consisting of a regulatory prodomain and two catalytic subunits, seems to be involved in the activation of both pathways (Wang et al., 2005). Activation of the mitochondrial independent apoptotic pathway is thought to occur through Ca^{2+}-dependent m-calpain cleavage of initiator caspase-12 (Nakagawa and Yuan, 2000). Once activated, caspase-12 cleaves caspase-9 directly, activating it in a cytochrome *c* and Apaf-1 independent fashion (Lamkanfi et al., 2002; Rao et al., 2002). Active caspase-9 in turn cleaves the effector caspase-3 and the apoptotic process continues as described above (Morishima et al., 2002; Rao et al., 2002). The interaction of caspase-12 with TRAF2 and its activation by proximity-induced dimerization followed by the signalling of the c-Jun N-terminal kinase (JNK) pathway (Yoneda et al., 2001), has been proposed to be the link between this caspase and the triggering of the mitochondrial-dependent apoptotic pathway (Shi, 2004). JNK is translocated to the mitochondrial membrane, and it stimulates the phosphorylation of Bim, which in turn is critical for Bax-dependent cytochrome c release (Lei and Davis, 2003; Putcha et al., 2003). Alternatively, caspase-12 may potentially interact with another pro-apoptotic protein, Bap31, a 28 kDa integral ER membrane protein containing a cytoplasmic domain that preferentially associates with caspase-8, Bcl-XL, and Bcl-2 (Groenendyk and Michalak, 2005). This interaction may be responsible for the feedback mechanism involving the

release of cytochrome *c* following the mitochondrial independent pathway activation by ER stress. Active caspase-8 can cleave Bap31, exposing the p20 amino-terminal transmembrane portion retained in the ER membrane. Cleaved Bap31 has been involved in induction of apoptosis via interaction with Drp1, a protein located at the mitochondria (Breckenridge et al., 2003). The ER-mitochondrial communication necessary for this process is possibly supported by physical links between the two organelles (Shoshan-Barmatz et al., 2004).

1.2.2.2.4 Caspase-independent apoptotic pathway

Some forms of cell death cannot be easily classified as apoptosis or necrosis. If caspases alone are responsible for the execution of apoptosis, cells should survive apoptotic treatments in the presence of caspase inhibitors such as Z-VAD fmk. However, Bax-triggered cell death, loss of mitochondrial membrane potential, release of cytochrome c and AIF were reported not to be inhibited by Z-VAD fmk (Green and Reed, 1998; Vermeulen et al., 2002; Xiang et al., 1996). These observations led to the idea of caspase-independent apoptosis.

Some authors have reported cases of programmed cell death with originally necrotic or non-apoptotic morphology. For example, TNF induces, depending on the cell type, apoptotic cell death with caspase activation or cell death with necrotic morphology and without caspase activation (Cauwels et al., 2003; Schulze-Osthoff et al., 1994). Studies of non-caspase proteases including cathepsins, calpains and serine proteases like granzyme A/B and Omi/HtrA2

suggest that the role of caspases in apoptosis is substitutable (Green and Kroemer, 2004; Johnson, 2000). These proteases can cooperate with caspases, but they can also trigger caspase independent apoptosis (Leist and Jaattela, 2001). The type of studies described above, have made evident that the classic dichotomy of apoptosis versus necrosis is a simplification of the way in which the organism disposes of unwanted and potentially harmful cells. Although caspases may be indispensable for the typical apoptotic morphology, the process of caspase activation is not the sole determinant of life and death decisions in programmed cell death.

Over the last 30 years, the characterization of the apoptotic process has increased significantly and so has our understanding of the underlying molecular mechanisms. However, many questions remain. For example, we still know very little about the activation mechanisms of the initiator caspases or how the Bcl-2 family of proteins controls the permeability of mitochondria and even less about the caspase- and mitochondria-independent cell death pathways. The apoptotic mechanisms will undoubtedly become more complex with the discovery and characterization of additional players, regulators and pathways. The evolutionary advantage of the existence of multiple death pathways is obvious: it protects the organism against the development of malignant diseases as many burdens have to be overcome before a cell becomes a tumour cell. This may explain the relative rarity of cancer, in respect to the huge number of cell divisions and mutations during a human life. The growing knowledge on the

mechanisms of spatial and temporal regulation of various signalling events involving cell death pathways is important for the oncology field, as they could potentially be manipulated to develop new cancer therapies.

1.3 Thesis objectives

The objective of this thesis is to explore the role of protein fatty acylation in the control of cellular metabolic processes. Specifically, we sought to determine the importance of palmitoylation in apolipoprotein B mediated lipoprotein particle assembly and secretion as well as the role of post-translational myristoylation of caspase-cleaved PAK2 during apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Materials

During the completion of this work, the following reagents and supplies were utilized. Unless otherwise stated, these products were used according to the instructions provided by the manufacturer.

Table 2.1 Reagents

Name	Source
β-mercapoethanol	Sigma-Aldrich
2-hyroxy-myristic acid (HMA)	Sigma-Aldrich
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Sigma-Aldrich
Acrylamide	Bio-Rad
Adenosine 5'-triphosphate (ATP)	Sigma-Aldrich
Agar	Difco
Agar	Invitrogen
Agarose, electrophoresis grade	Rose Scientific
Albumin	Pierce
Ammonium chloride (NH_4Cl)	Sigma-Aldrich
Ammonium persulphate	Bio-Rad
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
Bicinchoninic acid (BCA) Protein Assay Reagent	Pierce
Bis-acrylamide (N,N'- Methylene-bis-Acrylamide)	Bio-Rad
Bovine serum albumin (BSA) fatty acid free	Sigma-Aldrich
Bovine serum albumin (BSA) fraction V	Sigma-Aldrich
Bromophenol blue	BDH
Complete™ protease inhibitors	Roche
Coomassie Brilliant Blue R-250	ICN
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dimethylformamide (DMF)	BDH
Dithiobis(succinimidylpropionate) (DSP)	Pierce
Dithiothreitol (DTT)	Sigma-Aldrich
Dulbecco's modified Eagle's medium (DMEM)	Life Technologies
ECL Plus	Amersham Pharmacia
EN^{3}HANCE Surface Autoradiography Enhancer	PerkinElmer Life Sciences
Endoglycanase H (endo H)	New England BioLabs

Table 2.1 Reagents (continued)

Name	Source
Ethanol	Commercial Alcohols
Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N',N'-TetraAcetate (EGTA)	Fisher
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum (FBS)	Life Technologies
FuGENE 6 transfection reagent	Roche Applied Science
Gelatin	Fisher Scientific
Glacial acetic acid	Fisher
Glycerol	BDH
Glycine	Bio-Rad
Histone H1	Sigma-Aldrich
Höechst 33258	Sigma-Aldrich
Horse serum (HS)	Life Technologies
Hydrochloric acid	Fisher
ImageQuant software	Amersham Pharmacia
Immobilon-P PolyVinylidine Di-Fluoride (PVDF) membrane	Millipore Corporation
Isopropanol	Fisher
Leupeptin	Roche
L-methionine	Life Technologies
Low-Density Lipoprotein from human plasma, DiI complex (DiI-LDL)	Molecular Probes
Magnesium Chloride ($MgCl_2$)	Fisher
Malt extract	Difco
Mannitol	Sigma-Aldrich
Methanol	Fisher
Methionine/Cysteine-free DMEM	Life Technologies
MitoTracker Red CM-H_2Xros	Molecular Probes
Myristoyl-Coenzyme A	Sigma-Aldrich
N,N,N',N',-tetramethylenediamine (TEMED)	Invitrogen
N-Cyclohexyl-2-aminoethanesulfonic acid (HEPES)	Invitrogen
Nitrocellulose membrane (0.45 micron pore)	Bio-Rad
Nonidet P-40 (NP40)/IGEPAL CA-630	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Para-trifluoromethoxy carbonyl cyanide (FCCP)	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich

Table 2.1 Reagents (continued)

Name	Source
Phosphorimager screen	Amersham Pharmacia
Poly-L-lysine	Sigma-Aldrich
Potassium hydroxide (KOH)	Sigma-Aldrich
Potassium phosphate mono-basic (KH_2PO_4)	Sigma-Aldrich
Prolong Antifade Solution	Molecular Probes
Protein A-Sepharose CL-4B	Amersham Pharmacia
Protein G-Sepharose CL-4B	Amersham Pharmacia
Roswell Park Memorial Institute medium (RPMI) 1640	Invitrogen
Sepharose CL-4B	Sigma-Aldrich
Skim milk	Carnation
Sodium bicarbonate (Na_2CO_3)	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium citrate	Sigma-Aldrich
Sodium deoxycholate	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Bio-Rad
Sodium hydroxide (NaOH)	Sigma-Aldrich
Staurosporine (STS)	Sigma-Aldrich
Storm 840 phosphorimager	Amersham Pharmacia
Sucrose	BDH
Trichloroacetic acid (TCA)	Sigma-Aldrich
Tricine	Bio-Rad
Tris base	Roche
Triton X-100	BDH
Trypsin (0.25% w/v)	Life technologies
Tween 20 (polyoxyethylenesorbitan monolaureate)	Caledon
Urea	Bio-Rad
Yeast extract	Difco
Yeast Myristoyl-CoA:protein N-myristoyl transferase (NMT)	Berthiaume Laboratory

Table 2.2 Commonly Used Media and Buffers

Name	Composition
2.5X MS solution	525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl pH 7.4, 12.5 mM EDTA
2X Kinase Assay mix	0.4 mg/ml histone H1, 120 µM ATP, 10 µCi Redivue™ [γ-^{32}P]ATP, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 20 mM HEPES-NaOH pH 7.5, 1X Complete
2X Kinase buffer	20 mM $MgCl_2$, 10 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 40 mM HEPES-NaOH pH 7.5, 1X Complete
ATP stock solution	1.2 mM ATP, 1.2 mM $MgSO_4$ in 20 mM HEPES-NaOH pH 7.5
1X Blotto Solution	0.1% Tween 20, 150 mM NaCl, 50 mM Tris-HCl pH 7.5
CIP Extraction Buffer	300 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100, 0.1 mM DTT, 25 mM HEPES-NaOH pH 7.8 0.2 mM PMSF, 1X Complete
Coomasie Blue Gel Destaining solution	10% glacial acetic acid, 45% methanol in MQ water
Coomasie Blue Gel Staining solution	0.25% Coomasie Brilliant Blue R-250, 10% glacial acetic acid, 45% methanol in MQ water
DSP lysis buffer	50 mM HEPES-NaOH pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.15 M NaCl, 2% CHAPS, 1X Complete
DSP modified loading buffer	10% β-mercaptoethanol, 8 M urea, 10 mM Tris-Glycine pH 8.3, 2% SDS, 10% glycerol
DSP wash buffer	1% TX-100, 0.5 M NaCl in PBS pH 7.4., 1X Complete
Endo H denaturing buffer	0.5% SDS, 1% β-mercapoethanol,β-mercaptoethanol, 10 mM Tris-HCl pH 7.4
Endo H reaction buffer (10X)	500 mM sodium citrate pH 5.5
Histone H1 stock solution	20 mg/ml in 20 mM HEPES-NaOH pH 7.5
Laemmli 5X SDS-PAGE loading buffer	300 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.1% Bromophenol Blue, 1 M DTT or 5% β-mercaptoethanol
Laemmli Running Buffer	1% SDS, 0.025M Tris, 0.2M Glycine
Laemmli Separating Gel Buffer	0.4% SDS, 1.5M TrisHCl pH 8.3
Laemmli Stacking Gel Buffer	0.4% SDS, 0.5M TrisHCl pH 6.8

Table 2.2 Commonly Used Media and Buffers (continued)

Name	Composition
LB	1% tryptone, 0.5% yeast extract, 1% NaCl
Lipid Rafts lysis buffer	1% Triton X-100, 140 mM NaCl, 25 mM Tris-HCl pH 8.0, 1X Complete
Lysis buffer	
	50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM $MgCl_2$, 1X Complete
MgRS hypotonic buffer	10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 7.4, 1X Complete
NMT reaction buffer (2X)	20% sucrose, 4 mM EDTA, 40 mM DTT, 400 mM NaCl and 100 mM HEPES-NaOH pH 7.4
PBS	1.4 M NaCl, 30 mM KCl, 10 mM Na_2HPO_4-$7H_2O$, 14 mM KH_2PO_4
RIPA buffer	1mM EDTA, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1X Complete
TAE	40 mM Tris-acetate pH 8.0, 1 mM EDTA
TBS	140 mM NaCl, 25 mM Tris-HCl pH 7.5
TE buffer	10 mM Tris-HCl pH8.0, 1mM EDTA
Transfer Buffer	0.04M Glycine, 0.05M Tris, 20% methanol
Tris/Tricine 5X SDS-PAGE loading buffer	50 mM Tris-Glycine pH 8.3, 50% glycerol, 10% SDS, 0.1% Bromophenol Blue, 8 M urea, 5% β-mercaptoethanol
Tris/Tricine Anodic Buffer	0.2M Tris-HCl pH 8.9
Tris/Tricine Cathodic Buffer	0.1M Tricine, 0.1% SDS, 0.1M Tris-HCl pH 8.25
Tris/Tricine Separating Gel Buffer	3M Tris-HCl, 0.3% SDS, pH 8.45
Tris/Tricine Stacking Gel Buffer	0.41M Tris-HCl pH 7.5
Trypsin 2X sample buffer	0.25M Tris pH6.8, 8% SDS, 40% Glycerol, 0.1% Bromophenol Blue, 100 mM DTT, 1mM PMSF, 40.5 μM leupeptin

Table 2.3 Radiochemicals

Radiochemical	Source
[^{125}I]-Iodopalmitate	Berthiaume Laboratory (University of Alberta)
[9,10(n)-^{3}H]-Myristic acid	Amersham Biosciences
Redivue™ [γ-^{33}P] ATP	Amersham Biosciences
TRAN^{35}S-LABEL [^{35}S]-L-methionine	ICN Biomedicals

Table 2.4 Antibodies

Antibody	Application	Source	Catalog number
Chicken anti-rabbit IgG-Alexa Fluor 488	IF	Molecular Probes	A21442
Chicken anti-sheep IgG-Alexa Fluor 633	IF	Molecular Probes	A21100
Donkey anti-goat IgG-HRP	WB	Santa Cruz Biotechnology	sc-2020
Donkey anti-mouse IgG-Texas Red	IF	Jackson ImmunoResearch Laboratories	715-175-150
Donkey anti-rabbit IgG-HRP	WB	Amersham Biosciences	NA934-100UL
Goat anti-GFP	WB, IP	Eusera	EU4
Goat anti-mouse IgG-Alexa Fluor 488	IF	Molecular Probes	A21121
Goat anti-p-JNK (Thr 183/Tyr 185)	WB	Santa Cruz Biotechnology	sc-12882
Goat anti-rabbit IgG-FITC	IF	Jackson ImmunoResearch Laboratories	111-095-003
Goat anti-γPAK(C-19)	WB, IP	Santa Cruz Biotechnology	sc-1519
Mouse anti-human-apolipoprotein B (aa 474-579)	WB, IP	Heart Institute Research Corporation	1D1
Mouse anti-myc tag (4A6)	WB, IP	Upstate	05-724
Mouse anti-transferrin receptor	WB	Dr. Hanne Ostergaard (University of Alberta)	N/A
Phalloidin-Alexa Fluor 594	IF	Molecular Probes	A12381
Rabbit anti-calnexin	WB	StressGen Biotechnologies	SPA-865
Rabbit anti-caveolin	WB	BD Transduction Laboratories	610059
Rabbit anti-GFP	WB, IF	Eusera	EU2
Rabbit anti-giantin	WB	Dr. Ed Chan (University of California San Diego, USA)	N/A
Rabbit anti-JNK (C-17)	WB	Santa Cruz Biotechnology	sc-474
Rabbit anti-LIMK	WB	Cell Signaling	3842
Rabbit anti-myc tag	IP	Abcam	ab9106
Rabbit anti-p-LIMK	WB	Cell Signaling	3841

WB: western blot IP: immunoprecipitation IF: immunofluorescence HRP: horse radish peroxidase

Table 2.4 Antibodies (continued)

Antibody	Application	Source	Catalog number
Rabbit anti-rat IgG-FITC	IF	Jackson ImmunoResearch Laboratories	312-095-003
Rabbit anti-rat transferrin	IF	Biogenesis	9100-6009
Sheep anti-cytochrome c	IF	Sigma	C 5723
Sheep anti-human-apolipoprotein B	IP	Boehringer Mannheim	726 924
Sheep anti-mouse IgG-HRP	WB	Amersham Biosciences	NXA931-1ML

WB: western blot IP: immunoprecipitation IF: immunofluorescence HRP: horse radish peroxidase

Table 2.5 Antibiotics

Antibiotic	Source
Ampicillin	Calbiochem
Carbenicillin	Calbiochem
Cycloheximide	Sigma-Aldrich
Geneticin (G418 sulfate)	Life Technologies
Kanamycin	Calbiochem
Penicillin G (sodium salt)	Life Technologies
Streptomycin sulfate	Life Technologies

Table 2.6 DNA Modifying Enzymes

Enzyme	Source
Calf intestinal alkaline phosphatase (CIP)	New England BioLabs
Expand High Fidelity PCR system	Roche
Restriction Endonucleases	New England BioLabs
T4 DNA Ligase	New England BioLabs

Table 2.7 Plasmids used in this study

Plasmid	Source
pA^{213}C-t-PAK2-myc	This work
PAK2 cDNA	Origene (contained 5 point mutations)
pAla^{213}-N15-EGFP	Berthiaume Laboratory (University of Alberta)
pcDNA3.1/*myc*-His(-)A	Invitrogen
pEGFP-N1	Clontech Laboratories
pG^{213}C-t-PAK2-myc	This work
pGAP-43-GFP	Berthiaume Laboratory (University of Alberta)
pGly^{213}-N15-EGFP	Berthiaume Laboratory (University of Alberta)

Table 2.8 Primers used in this study

Primer title	Primer sequence (5' → 3')	Engineered site	Use
51Sf2	gtaggagatgaattgtttgtggtcatg	N/A	C-t-PAK2 sequencing primer forward
51Sr3	ctccaatgcctgtaaacactctc	N/A	C-t-PAK2 sequencing primer reverse
BSmP3	cggggatccttacaggtcttcttccgatatgagttt ctgctcacggttactcttcattgcttctttag	*BamHI*	G^{213} and A^{213}C-t-PAK2-myc reverse primer
EKSP5-A	gaggaattcgccaccatggctgctgccaagtct ttagacaaac	*EcoRI*	A^{213}C-t-PAK2-myc forward primer
EKSP5-G	gaggaattcgccaccatgggtgctgccaagtct ttagacaaac	*EcoRI*	G^{213}C-t-PAK2-myc forward primer
G413Ef	atactggatggcaccagaggtggttacacgga aag	N/A	G→E mutagenesis of PAK2 cDNA forward primer
G413Er	ctttccgtgtaaccacctctggtgccatccagtat	N/A	G→E mutagenesis of PAK2 cDNA reverse primer
V451Af	atgaaaatcccttgagggtcttgtacctaatagc aac	N/A	V→A mutagenesis of PAK2 cDNA forward primer
V451Ar	gttgctattaggtacaagaccctcaagggattttc at	N/A	V→A mutagenesis of PAK2 cDNA reverse primer

Table 2.9 Cell Lines used in this study

Cell	Description	Source
Jurkat	Human, peripheral blood, leukemia, T cell	Dr. Michele Barry (University of Alberta)
COS-7	African green monkey, kidney cell	ATCC
McArdle RH-7777	Rat liver, hepatoma cell	Dr. Zemin Yao, University of Ottawa
McArdle RH-7777 WT apoB-29	Rat liver, hepatoma cell, stably expressing wild type human apoB-29	Berthiaume Laboratory (University of Alberta)
McArdle RH-7777 (C1085S) apoB-29	Rat, liver, hepatoma cell, stably expressing (Cys1085Ser) human apoB-29	Berthiaume Laboratory (University of Alberta)

2.2 Methodology

2.2.1 Molecular cloning and transfection

Sequencing of the purchased PAK2 cDNA showed that it contained 2 point mutations at the carboxy-terminal end of the protein. The mutations were corrected using the Quick-Change mutagenesis kit (Stratagene) by Andrea Seime and made to conform to the human PAK2 nucleotide sequence reported to the Entrez Nucleotide Data Bank at NCBI with accession number BC069613.

The myristoylatable Gly^{213}-C-t-PAK2-myc and the non-myristoylatable A^{213}-C-t-PAK2-myc constructs were engineered using PCR. The reactions were performed in a total volume of 50µl containing 100 ng of corrected PAK2 cDNA as template, 20 pmol of EKSP5-G or EKSP5-A forward primer, 20 pmol of BSmP3 reverse primer 3.5 U of Expand High Fidelity DNA Polymerase, 2.5 mM of each dNTP and 5 µl of 10X Expand High Fidelity Buffer . The reactions were amplified for 30 cycles in a MJ Thermocycler (MJ Research) as follows:

Cycle 1: 94 °C for 2 min.

Cycles 2 to 31: 94 °C for 1 min, 50 °C for 1min, 72 °C for 1 min.

Cycle 32: 72 °C for 7 min, 4°C forever.

The PCR products were cleaned from proteins and dNTPs using the QIAquick PCR purification kit (QIAGEN) before restriction endonuclease cleavage.

The pcDNA3.1/myc-His (-) A mammalian expression vector was digested with the appropriate restriction endonucleases and treated with CIP (as

recommended by the manufacturer) in order to remove the 5′ phosphates and therefore prevent its re-circularization during ligation. The digested PCR products as well as the de-phosphorylated, linearized vector were separated by electrophoresis on a 1% TAE-agarose gel at 80 V and, after visualization by ethidium bromide staining; the corresponding DNA fragments were purified from the gel using the QIAEX II Gel Extraction Kit (QIAGEN) as instructed by the manufacturer.

The extracted PCR fragments and vector were combined in a 4:1 molar ratio (400-500 ng total DNA) and ligated overnight in a 50 µl final volume reaction at 16 °C using T4 DNA ligase following manufacturer's specifications.

The ligation products were introduced into competent *E. coli* DH5α by chemical transformation. Briefly, a 200 µl aliquot of chemically competent *E. coli* DH5α was thawed on ice and combined with 10 – 25 µl (100-250 ng) of ligation reaction. The mixture was incubated for 30 min in ice, subjected to a 42 °C heat shock for 90 seconds and placed back on ice for a 2 min recovery period. The cells were then incubated with 800 µl of LB broth for 45 min at 37 °C, spread on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37 °C.

Five to ten colonies from the Gly^{213}-C-t-PAK2-myc and A^{213}-C-t-PAK2-myc ligation plates were re-streaked in fresh antibiotic-containing LB agar dishes and also inoculated into 5 ml LB broth containing 50 µg/ml carbenicillin. The liquid cultures were grown 16 h at 37 °C with agitation. Plasmid DNA was purified from the cell cultures using the QIAprep Spin Miniprep Kit (QIAGEN) as

indicated by the manufacturer. The presence of the PCR fragment was assessed by restriction endonuclease digestion of the plasmids followed by agarose gel electrophoresis and visualization by ethidium bromide staining. Plasmids yielding the expected restriction band pattern were fully sequenced using automated DNA sequencing (DNA Core Facility, University of Alberta) in order to verify whether they conformed to the original design.

Bacterial colonies harboring the desired plasmids were transferred to 6 ml LB cultures containing 50 μg/ml carbenicillin and incubated at 37 °C for 8 h with agitation. The cultures were transferred to 1 l sterile LB with antibiotic and further incubated at 37 °C for 16 h with agitation (300 RPM). After incubation an 800 μl aliquot from each culture was used to make a glycerol stock. The rest of the cultures were harvested by centrifugation at 5000 xg for 10 minutes and the plasmid DNA purified using the UltraMobius™ 1000 Plasmid Kit (Novagen) as specified by the manufacturer.

Plasmids were transfected into COS-7 cells using FuGENE 6 transfection reagent at a 3 μl FuGENE6: 2 μg DNA ratio as indicated by the manufacturer. Cells were always assayed no later than 24 h post-transfection.

2.2.2 Cell lines maintenance

McArdle RH-7777 rat hepatoma cells stably expressing human wild type apoB-29 and human (Cys1085Ser) apoB-29 were grown in DMEM containing 100 U/ml penicillin G (sodium salt), 100 mg/ml streptomycin sulfate, 400 μg/ml geneticin and supplemented with 10% FBS and 10% HS (Zhao et al., 2000).

Jurkat T cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin G (sodium salt) and 100 mg/ml streptomycin sulfate.

COS-7 cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin G (sodium salt) and 100 mg/ml streptomycin sulfate.

All cell lines were maintained at 37 °C in a humidified incubator with a 7% CO_2 atmosphere and passaged regularly.

2.2.3 Metabolic labeling of cells

Approximately 3.25×10^6 McArdle RH-7777 rat hepatoma cells stably expressing either wild type human apoB-29 or human (C1085S) apoB-29 were seeded onto 100 mm dishes and grown overnight as described above. Cells were washed twice with methionine/cysteine-deficient DMEM and incubated with 2.5 ml of methionine/cysteine-deficient DMEM containing 280 µCi of Tran^{35}S Label for 15 min or incubated for 1 h with DMEM supplemented with 10 µg/ml fatty acid free BSA and then pulsed for 15 minutes with 250 µCi of [^{125}I]iodopalmitate in 2.5 ml DMEM supplemented with 10 µg/ml fatty acid free BSA (Berthiaume and Resh, 1995). After incubation and removal of pulse medium, the cells were quickly washed twice with warm PBS, and 4 ml of DMEM supplemented with 200 µM methionine or 2.5 ml of DMEM supplemented with 10 µg/ml fatty acid free BSA were added. Cells were incubated at 37 °C for 0, 15, 30, 45, 60, 90, 120 and 240 minutes.

Jurkat T cells were metabolically labeled in RPMI 1640 without serum containing potassium [9,10(n)-^{3}H]myristate (1 mCi/1x10^7 cells) and 0.1% (w/v) fatty acid-free BSA for 1 h at 37 °C in a humidified incubator with a 7% CO_2

atmosphere. To induce apoptosis, cells were treated for 5 h with vehicle or 2.5 μM staurosporine (STS) and 5 μg/ml cycloheximide (CHX) (Iglesias-Serret et al., 2003; Seghatchian and Krailadsiri, 2001; Vilas et al., 2006). In N-myristoylation inhibition studies, 1 hr prior to metabolic labeling, medium containing 0.1% (w/v) fatty acid-free BSA was supplemented with 1 mM 2-hydroxymyristic acid (HMA) which was left in for the duration of the incubation.

COS-7 cells were transfected with either Gly^{213}-C-t-PAK2-myc, A^{213}-C-t-PAK2-myc, pEGFP-N1 or pGAP-43-GFP constructs. At 12-14 h post transfection the cells were starved for 1 h in serum-free DMEM and then metabolically labeled in DMEM containing potassium [9,10(n)-^{3}H]myristate (400 μCi/ml) and 0.1% (w/v) fatty acid-free BSA (C-t-PAK2-myc constructs) or potassium [^{125}I]palmitate (40 μCi/ml) and 0.01% (w/v) fatty acid-free BSA (pEGFP-N1 and pGAP-43-GFP) for 4 h at 37 °C in a humidified incubator with a 7% CO_2 atmosphere.

2.2.4 Immunoprecipitations

ApoB. At the indicated times, the chase medium was recovered from the labeled McArdle RH-7777 cells and lipoproteins were solubilized for 24 h at 4 °C in the presence of 0.1% SDS-RIPA buffer. Cells were then lysed with hot (75 °C) 1% SDS-RIPA buffer, solubilized 24 h at 4 °C and diluted to a final concentration of 0.1% SDS in RIPA buffer (Vilas and Berthiaume, 2004). After solubilization, an aliquot was removed in order to determine the amount of total proteins present in each sample. ApoB lipoproteins were collected from the media or cell lysates by immunoprecipitation using Roche's anti-human apoB

polyclonal serum (20 µl, 2 h at 4 °C with agitation) or mouse monoclonal 1D1 anti-human apoB (8 µg, 3 h at 4 °C with agitation). In immunoprecipitations, 100 µl of a slurry made of 20% (v/v) Protein A-Sepharose CL-4B and 30% (v/v) Sepharose CL-4B was added to solubilized supernatants and cell lysates and incubated for 2 h at 4 °C with agitation. Tubes containing the reactions were centrifuged for 15 min at 1000 xg to isolate the Sepharose beads containing the immune complexes. The beads were further washed 5 times with 0.1% SDS-RIPA buffer. In order to elute the apoB, the beads were heated at 75 °C for 15 min in the presence of either endo H denaturing buffer or Tris/Tricine SDS-PAGE loading buffer, spun down 1 min at maximum velocity in a table-top micro centrifuge and the supernatants were recovered.

PAK2. Metabolically radio-labeled Jurkat and COS-7 cells were collected and lysed in cold lysis buffer for 30 min at 4 °C. Lysates were clarified at 1000 xg for 10 min in a refrigerated centrifuge. PAK2 was immunoprecipitated from clarified Jurkat lysates with goat anti-γPAK(C-19) antibody (3 µg/ 200 µg total protein) for 4 h. Myc-tagged proteins and GFP-tagged proteins were immunoprecipitated from clarified COS-7 lysates using rabbit anti-myc antibody (1:250) overnight or goat anti-GFP antibody (1 µg/ml) for 2 h, respectively. Immunocomplexes were pulled down with 50 µl protein G–Sepharose (50% v/v) for 2 h. All the steps were carried out at 4 °C with gentle rocking. The Protein G-Sepharose beads were washed three times with cold lysis buffer.

2.2.5 Electrophoresis and western blotting

Protein samples from McArdle RH-7777 rat hepatoma cells were separated using discontinuous Tris-Tricine polyacrylamide gels made of a 3% percent acrylamide stacking gel in which there are 3% Total monomers and 3% Crosslinkers (3%T, 3%C) and a 5.5% acrylamide separating gel in which there are 5.5% Total monomers and 3% Crosslinkers (5.5%T, 3%C) (Schagger and von Jagow, 1987).

The remaining portion of the protein samples in this work were separated using regular discontinuous Tris-Glycine polyacrylamide gels with 3%T, 3%C stacking gels and separating gels of different T percentages ranging from 8 to 12.5 % and 3%C (Sambrook and Russell, 2001), based on the separation requirements of the experiments.

After electrophoresis, proteins were transferred onto Immobilon-P PVDF membranes for 2 hours at 0.8 A in a Bio-Rad Trans Blot apparatus. For immunodetection, membranes were blocked for 1 h at room temperature with 0.1% Tween 20 in Blotto containing either 5% skim milk or 3% BSA with gentle rocking. After incubation with the corresponding primary antibodies, the membranes were subjected to 3 cycles of washes as follows: 2 washes of 5 min each with cold PBS, 2 washes of 5 min each with cold 0.1% Tween 20 in PBS and 2 washes of 5 min each with cold PBS. After the washes the membranes were incubated with the corresponding HRP-conjugated secondary antibodies, washed as described above and processed for ECL detection.

2.2.6 Detection and analysis of immuno-complexes

2.2.6.1 ApoB-29 secretion analysis

Aliquots of the apoBs eluted from the immune complexes originating from radiolabeled cell lysates and media were separated by electrophoresis (Schagger and von Jagow, 1987), dried and exposed to a phosphorimager screen. Intensities of the bands corresponding to apoB-29 obtained on a Storm 840 phosphorimager were quantified using the ImageQuant software (Amersham Pharmacia, Uppsala, Sweden). The values obtained were normalized by the total amount of proteins at each given time and then expressed as percentage of total arbitrary units at t=0.

2.2.6.2 Detection of PAK2 and GFP constructs

Immuno-complexes were separated by SDS-PAGE (12.5%). Gels containing [^{3}H]myristate- or [^{125}I]iodopalmitate-labeled samples were transferred onto PVDF membranes to allow immuno-detection of PAK2 or GFP using goat anti-γPAK(C-19) (1:1000) and donkey anti-goat-HRP (1:5000) or rabbit anti-GFP (1:10000) and donkey anti-rabbit-HRP (1:10000) respectively. After detection, [^{3}H]myristate-containing PVDF membranes were washed 3 times in cold PBS and either air-dried for 16 h, treated with EN^{3}HANCE Surface Autoradiography Enhancer and exposed to a Biomax MS film for 30-45 d at –80°C or to a phosphorimager screen for 10 d at room temperature. [^{125}I]iodopalmitate-labeled proteins were detected using a phosphorimager screen.

2.2.7 Quantitation of apoB-29 bound to calnexin using DSP cross-linking

The cross-linking experiments using the reducible cross-linker DSP were performed as described by Linnik (Linnik and Herscovitz, 1998) except that DSP was used at a 10 times higher concentration and that all buffers contained freshly added 1 mM PMSF and 20 μg/ml Leupeptin. Approximately 3.0 x 10^6 McArdle RH-7777 rat hepatoma cells stably expressing either wild type human apoB-29 or human (C1085S) apoB-29 were seeded onto 100 mm dishes, grown overnight and incubated 1 h in the presence of methionine/ cysteine-deficient DMEM, pulsed with 200 μCi/plate of Tran^{35}S Label for 15 min and chased for 0, 15, 30, 45 and 60 min as described in 2.2.3. At each time point, the cells were washed twice with cold PBS and incubated with 3 ml of a 2 mM solution of DSP in PBS (pH 7.4) for 2 h on ice. The cross-linking reaction was stopped by addition of 150 μl of 1M Tris-HCl pH 7.4 for 10 min on ice.

Cells were lysed with 1 ml of DSP lysis buffer and the lysates were pre-cleared with 100 μl of 50% slurry of protein A-Sepharose in water for 45 min at 4 °C with agitation. After centrifugation (2 min at 16,000 xg), CLX was immunoprecipitated from the supernatants using with 8 μl of a rabbit anti-CLX antibody for 4 h at 4 °C with agitation. Immune complexes were recovered by incubation with 100 μl of 50% slurry of protein A-Sepharose in water for 3 h at 4 °C with agitation. The beads were recovered by centrifugation and washed three times with DSP lysis buffer, three times with DSP wash buffer and three times

with PBS, 1X Complete (Linnik and Herscovitz, 1998) and CLX with its cross-linked proteins were recovered by boiling the beads 5 min at 100 °C in 200 µl of 1% SDS in PBS pH 7.4. The supernatant was recovered by centrifugation and diluted 5 fold with 1% TX-100 in PBS pH 7.4 and apoB-29 recovered by immunoprecipitation with 20 µl of 1D1 monoclonal antibody overnight at 4 °C with agitation followed by protein A-Sepharose treatment as described above and washed three times with 1 ml of DSP wash buffer.

To release apoB-29 from CLX, the DSP cross-linker was reduced by boiling the beads 5 min at 100 °C in 100 µl of 1X DSP modified loading buffer containing 10% β-mercaptoethanol. The samples were separated and transferred to a PVDF membrane as described in 2.2.5. In order to quantify and confirm the presence of total CLX and apoB-29 in each lane, the bands corresponding to CLX and apoB-29 were identified by western blot/ECL using a rabbit anti-CLX antibody and 1D1 monoclonal antibody respectively (not shown). The ECL signal was then quenched by drying the membranes overnight and the dried membranes were exposed to a phosphorimager screen in order to quantify the ^{35}S-labeled apoB-29 released from CLX. The relative specific activity (^{35}S-signal/ECL signal) of each apoB-29 band was calculated and plotted.

2.2.8 Endoglycosidase H cleavage assay

McArdle RH-7777 rat hepatoma cells stably expressing either wild type human apoB-29 or human (C1085S) apoB-29 were incubated 1 h in the presence of methionine/ cysteine-deficient DMEM, pulsed with 200 µCi/ plate of

Tran^{35}S Label and chased for 0, 15, 30, 45, 60, 90, 120 and 240 min as described in 2.2.3. At each time point apoB was immunoprecipitated from media and cell lysates with 20 µl of 1D1 monoclonal antibody O.N. at 4°C with agitation followed by incubation with 100 µl of 50% slurry of protein A-Sepharose in water for 3 h at 4 °C with agitation and combined. ApoB was eluted from the protein-A Sepharose by boiling the beads 5 min at 100 °C in 200 µl of 1% SDS in PBS pH 7.4. The supernatants were recovered by centrifugation and diluted 5 fold with 1% TX-100 in PBS pH 7.4 and incubated overnight at 37 °C in the presence of 500U of endo H in 1X endo H reaction buffer. The digestion products were then separated by electrophoresis, apoB was immunodetected and the relative specific activity (^{35}S-signal/ECL signal) activity of each apoB-29 band was calculated. The data were expressed as percentage of endo H-sensitive apoB-29 in arbitrary units.

2.2.9 Sub-cellular fractionation

2.2.9.1 Microsomal purification

Monolayers of McArdle RH-7777 cells either expressing wild type human apoB-29 or human (Cys1085Ser) apoB-29 were harvested, swelled in 250 µl of MgRS hypotonic buffer for 15 min on ice and disrupted in a Dounce homogenizer (20 strokes with a tight pestle). Following the addition of 0.4 volumes of 2.5 x MS solution to preserve organelle integrity, the cells were homogenized 5 more times. The post-nuclear supernatant (1000 xg) was further

centrifuged at 16,000 xg for 20 min at 4 °C yielding a membranes and microsome-enriched pellet.

2.2.9.2 Fractionation of STS and HMA treated cells

Appropriately transfected COS-7 cells or Jurkat T cells previously incubated for 5 h in RPMI 1640 without serum at 37 °C in a humidified incubator with a 7% CO_2 atmosphere with vehicle or 2.5 μM STS, 5 μg/ml CHX with 0.1% (w/v) fatty acid-free BSA alone or supplemented with 1 mM HMA were fractionated into a soluble, cytosolic fraction (S) and an insoluble fraction containing mostly cellular membranes (P) (Berthiaume et al., 1994). Cells were harvested, swelled in 250 μl of MgRS hypotonic buffer for 15 min on ice and disrupted in a Dounce homogenizer. Following the addition of 0.[illegible] of 2.5 x MS solution, the cells were homogenized five more times. The homogenates were centrifuged at 1000 xg for 5 min at 4 °C to pellet nuclei and intact cells. The post-nuclear supernatant (S1) was recovered and the pellet disrupted five more times in 1x MS solution. The homogenate was centrifuged once more at 1000 xg for 5 min at 4 °C and the supernatant obtained (S2) pooled with S1. Half of the pool was kept (T) and total protein content was determined in it. Finally, the remaining of the pooled S1+S2 fractions were centrifuged at 100,000 xg for 30 min at 4 °C yielding a cytosolic-enriched supernatant (S) and a membrane-enriched pellet (P). Equal amounts of total protein (T) and the corresponding volume of S and P fractions for each treatment were combined with the appropriate amount of Laemmli 5X SDS-PAGE loading buffer, boiled for 5 min, and separated by SDS-

PAGE (12.5%). Gels were transferred onto PVDF membranes and PAK2 was immuno-detected as described above.

2.2.10 Permeabilization of microsomes by Na_2CO_3 treatment and trypsin digestion of microsomal extracts

Microsomes were permeabilized in cold 100 mM Na_2CO_3 pH 11.0 at a concentration of 2 mg protein/ml, incubated on ice for 15 min and centrifuged at 120,000 xg for 10 min at 4 °C in a Beckman TLA 100.4 rotor (Beckman Instruments) (Michalak et al., 1991). The supernatant was diluted in 1 x MS solution, separated into 1 mg aliquots, frozen in liquid nitrogen and stored at –80 °C. Sodium carbonate treatment does not affect the integrity of lipoprotein particles and is routinely used to prepare microsomal extracts containing lipoprotein particles prior to density gradient analysis in VLDL secretion experiments (Boren J et al., 1992; Boren et al., 1993; Stillemark P et al., 2000).

Sodium carbonate supernatant aliquots (1 mg) were thawed on ice and adjusted to pH 8.0 with 1 M KH_2PO_4 pH 4.0. The aliquots were incubated with 10 µg of trypsin at 37 °C, and at various times 0.1 mg aliquots were taken out. Reactions were stopped by the addition of one volume of Trypsin 2X sample buffer and frozen immediately.

2.2.11 Fractionation of cellular membranes on sucrose gradient and analysis of apoB-29 content using slot blot

Fractionation of cellular membranes was carried out using the established procedure of Evans (Evans, 1992). Microsomes form McArdle RH-7777 cells either expressing wild type human apoB-29 or human (Cys1085Ser) apoB-29 were resuspended in 1 ml of a 10% (w/v) sucrose solution in 10 mM Tris-HCl pH 7.4 containing 20 µg/ml of leupeptin and layered on top of a discontinuous sucrose gradient consisting of (from bottom to top) 1.5 ml of 70% (w/v), 2 ml of 55% (w/v), 5 ml of 39% (w/v), 2 ml of 27% (w/v) sucrose in 10 mM Tris-HCl pH 7.4. The gradient was then topped with 0.5 ml of buffer and centrifuged at 100,000 xg in a Beckman SW 41 Ti rotor (Beckman Instruments) at 4 °C for 5 h. After centrifugation, 1 ml fractions were collected (from top to bottom) and PMSF was added to a 1 mM final concentration. Twenty micro liter aliquots of each fraction were diluted to 1 ml in 10 mM Tris-HCl pH 7.4 containing 1 mM PMSF, 20 µg/ml leupeptin, and blotted onto a 0.45 micron nitrocellulose membrane (previously equilibrated for 10 minutes in the same buffer) using a Bio-Rad Slot Blot Apparatus (Bio-Rad). After blotting, membranes were blocked for 1 h in a 5% milk-Blotto solution and probed for human apoB using the 1D1 anti-human apoB as described earlier. Mouse 1D1 monoclonal antibody does not cross-react with endogenous rat apoB (Zhao et al., 2000).

2.2.12 Immunocytochemistry and live cell fluorescence microscopy

For immunocytochemistry, McArdle RH-7777 cells stably expressing either wild type human apoB-29 or (Cys1085Ser) apoB-29 or appropriately transfected COS-7 were grown in six well tissue culture plates each one containing a glass coverslip (no.1 thickness, Fisher Scientific) previously coated with 5 μg/ml poly-L-lysine. Cells were washed twice in PBS, fixed in 4% paraformaldehyde in PBS pH 7.4, for 20 min, washed twice with PBS and then processed as follows:

For immunocytochemistry of McArdle RH-7777, cells were blocked 1 h with 4% normal donkey serum in PBS at room temperature and coverslips were incubated with the appropriate antibodies, diluted in 4% normal donkey serum in PBS to prevent non-specific binding, for 1 h at 37 °C in a humidified chamber. To detect apoB-29 and transferrin, 1D1 mouse monoclonal anti-human apoB and rat anti-transferrin antibodies both at a 1:1000 dilution were used respectively. After incubation, cells were washed 4 times with PBS and further incubated with donkey anti-mouse-Texas Red and rabbit anti-rat-FITC (1:200) for 1 h at 37 °C.

For immunocytochemistry of COS-7, cells were quenched with 50 mM NH_4Cl for 10 min and permeabilized with 0.1% Triton X-100 in PBS pH 7.4 for 1 min at room temperature, followed by a 30 min block with 0.2% gelatin in PBS pH 7.4. For colocalization of myc chimeras, various organelles and cellular structures, cells were incubated for 1 hour at 37 °C in a humidified chamber with

mouse monoclonal anti-myc tag clone 4A6 (1:500), phalloidin-Alexa Fluor 633 or 594 (3 units/coverslip), DiI-LDL (1 mg/ml) and Hoechst 33258 (50 ng/ml) to detect the myc epitope, actin cytoskeleton, endosomes and nuclei respectively. Goat anti-mouse-Alexa Fluor 488 (1:1000) was used to detect anti-myc antibodies. Mitochondrial potential was detected with MitoTracker Red CM-H2XRos (1 μM) as recommended by the supplier. Cytochrome c was detected using sheep anti-cytochrome c antibody (1:1000) followed by chicken anti-sheep-Alexa Fluor 633 (1:200). All antibodies used were diluted in 0.2% gelatin in PBS pH 7.4. DiI-LDL was added to living transfected cells 1 h before fixation to allow for incorporation of the fluorescent lipoprotein particle into endosomes.

After processing, all coverslips were mounted in Prolong Antifade Solution and observed by confocal laser scanning microscopy using appropriate filter sets.

For live cell fluorescent microscopy, COS-7 cells co-transfected with the various PAK2 constructs or empty vector and EGFP as transfection marker were grown in six well tissue culture plates and analyzed at 12, 16, 20 and 24 h post-transfection. At each time point cells were incubated for 15 min with 25 μg/ml of the permeable nuclear dye Höechst 33258, washed twice with warm PBS and kept in warm 0.1% FBS in PBS pH 7.4. Eight to twelve randomly selected fields, representing 150-300 transfected cells, were photographed with a Nikon epifluorescence microscope using bright field, FITC and UV filters. Since the expression of C-t-PAK2 leads to adherent cell shrinking and rounding up without exhibiting classical hallmarks of apoptosis (no phosphatidylserine

externalization and absence of TUNEL reactivity), the percentage of cells undergoing programmed cell death was calculated as the percentage of green cells presenting condensed/fragmented nuclei and rounded/retracted/highly refractive morphology as described in Lee *et al.* (Lee et al., 1997) and, Rudel and Bokoch (Rudel and Bokoch, 1997). Alternatively, COS-7 cells grown on poly-L-lysine coated cover slips were transfected with EGFP chimeras either containing the first 15 amino acids of C-t-PAK2 (Gly^{213}-N15-EGFP), a non-myristoylatable polypeptide Ala^{213}-N15-EGFP or vector alone. Twelve to fourteen hours post-transfection, coverslips were removed from media, washed with warm PBS, mounted on glass slides in 0.1% FBS in PBS pH 7.4 with the use of vacuum grease or nail polish as a sealant and EGFP natural fluorescence was detected by confocal laser scanning microscopy using appropriate filter sets.

2.2.13 *In vitro* myristoylation and mass spectrometry of PAK2 synthetic peptides

The following peptides were synthesized at Service de Synthese de Peptides de L'Est du Quebec, Le Centre Hospitalier de L'Universite Laval: GAAKSLDKQK (Gly^{213}-N10-C-t-PAK2), AAAKSLDKQK (Ala^{213}-N10-C-t-PAK2) and myristoyl-GAAKSLDKQK (myr-N10-C-t-PAK2) which was used as a positive myristoylation control.

The lyophilized peptides were reconstituted in DMSO at a final concentration of 1 mM. The peptides (Gly^{213}- or Ala^{213}-N10-C-t-PAK2; 50 μM)

were incubated with 25 µl of 2X NMT reaction buffer in presence or absence of 50 µM of myristoyl-Coenzyme A and 1 µg of N-Myristoyltransferase in a final volume of 50 µl for 30 min at room temperature. The reaction was then stopped by TCA precipitation and the supernatants containing the peptides were analyzed by liquid chromatography and then MS at the Institute for Biomolecular Design (University of Alberta).

2.2.14 Characterization of the proteinyl-fatty acyl linkage

PVDF membranes containing [^{3}H]myristate- or [^{125}I]palmitate-labeled samples were treated as described previously (Armah and Mensa-Wilmot, 1999; Berthiaume and Resh, 1995). PVDF membranes containing [^{3}H]myristate- or [^{125}I]iodopalmitate-labeled proteins were soaked in 200 ml of either 0.2 N KOH or 1 M Tris-HCl, pH 7.0 at 25 °C with shaking. The solutions were replaced every 30 min for 1.5 h and then the membranes were incubated in 500 ml of the same solution for 22.5 h at 25 °C with shaking . After incubation, the membranes were rinsed 3 times with PBS, air dried and [^{3}H]myristate- or [^{125}I]palmitate-radiolabeled proteins were detected by fluorography using the appropriate phosphorimager screens.

2.2.15 Isolation of lipid raft microdomains by sucrose-gradient centrifugation

COS-7 cells grown in 100 mm plates were transiently transfected with either Gly^{213}-C-t-PAK2-myc or the non-myristoylatable A^{213}-C-t-PAK2-myc

chimeras. Fourteen to sixteen hours post transfection the cells were washed with ice-cold PBS and harvested in 1 ml of cold Lipid Rafts lysis buffer. The samples were then separated in two equal aliquots and incubated for 30 min on ice or at 37 °C. After incubation, the aliquots were further disrupted in Dounce homogenizers (10 strokes with a tight pestle) kept at the same temperature as the samples. The samples were then centrifuged at 1000 xg for 10 min in a refrigerated centrifuge. The supernatants were rapidly adjusted to 40% (w/v) sucrose by addition of 1 ml of cold 80% (w/v) sucrose prepared in 25 mM Tris-HCl pH 8.0, loaded into a 12.5 ml ultracentrifuge tube and placed on ice. A 5–40% discontinuous sucrose gradient was formed above the homogenates by adding 7.5 ml of cold 30% (w/v) sucrose in 25 mM Tris-HCl pH 8.0 followed by 2.5 ml of cold 5% (w/v) sucrose in the same buffer (Radeva and Sharom, 2004).

Samples were centrifuged at 120,000 xg for 16 – 20 h at 4 °C using a SW40 Ti rotor. Fractions of 1 ml (typically 12–13 fractions in total) were collected from the top of the gradient tube using a P-1000 Gilson Pipetman pipette and TCA precipitated. The pellets were solubilized in 50 µl of 1X Laemmli SDS-PAGE loading buffer and analyzed by SDS-PAGE followed by western blot. PAK2, the lipid raft marker caveolin and the non-raft protein transferrin receptor were immunodetected using goat anti-γPAK(C-19) (1:1000), rabbit anti-caveolin (1:5000) and mouse anti-transferrin receptor (1:1000) respectively.

2.2.16 *In vitro* C-t-PAK2-myc kinase activity assay

COS-7 cells grown in 100 mm plates were transiently transfected with either empty vector, Gly^{213}-C-t-PAK2-myc or the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras. Fourteen to sixteen hours post transfection the cells were washed with ice-cold PBS, lysed in 1 ml of cold Lipid Rafts lysis buffer supplemented with 1 mM Na_3VO_4 and 50 mM NaF and left 30 min on ice. The samples were then disrupted by passing them 10 times through a 26 G x ½" needle and then centrifuged at 1000 xg for 10 min at 4 °C. The supernatants were recovered and adjusted to contain equal amounts of total proteins. C-t-PAK2-myc was immunoprecipitated using rabbit anti-myc antibody (1:250) and recovered from the lysates using protein G-sepharose beads as described above.

The beads containing the immunocomplexes were washed twice with cold Lipid Rafts lysis buffer supplemented with 1 mM Na_3VO_4 and 50 mM NaF to inhibit the possible action of unspecifically associated phosphatases, twice with 1X Kinase buffer and finally resuspended in 20 µl of the same buffer. The beads were then combined with 20 µl of 2X Kinase Assay mix and incubated for 30 min at 30 °C. The reactions were stopped by addition of 10 µl of Laemmli 5X SDS-PAGE loading buffer and immediate boiling for 5 min. The samples were separated by SDS-PAGE (15%), and the gel was then stained, destained, dried and exposed to a phosphorimager cassette.

2.2.17 Dephosporylation of C-t-PAK2-myc chimeras with CIP

COS-7 cells grown in 100 mm plates were transiently transfected with either empty vector, Gly^{213}-C-t-PAK2-myc or the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras. Fourteen to sixteen hours post transfection the cells were washed with ice-cold PBS, lysed in 250 µl of cold CIP Extraction Buffer and incubated 30 min on ice. The samples were then disrupted by passing them 10 times through a needle and then centrifuged at 1000 xg for 10 min at 4 °C. Supernatants were recovered and 50 µg of total protein from each sample were diluted to 48 µl with 1X NEB buffer #5. Samples were then incubated for 1 h at 37°C with either 2 µl (20 U) of CIP or water. The reactions were stopped by addition of 12 µl of Laemmli 5X SDS-PAGE loading buffer and 50 mM EDTA followed by 5 min boiling. Samples were separated by SDS-PAGE (12.5%), and transferred onto PVDF membranes. PAK2 was immunodetected using anti-γPAK(C-19) (1:1000) as described above.

CHAPTER 3

A role for palmitoylation in the quality control, assembly and secretion of apolipoprotein B

A version of this chapter has been published in Gonzalo L. Vilas and Luc G. Berthiaume. "A role for palmitoylation in the quality control, assembly and secretion of apolipoprotein B". *Biochem. J.* (2004) **377**, 121-130.

3.1 Overview

ApoB-100 and apoB-48 are important components of low-density lipoprotein particles (LDL), intermediate density lipoprotein particles (IDL) and both very low-density lipoprotein particles (VLDL) and chylomicrons remnants (Davis, 1996; Havel, 1995). Interestingly, increased levels of all these apoB-containing lipoprotein particles are considered to be atherogenic since their plasma concentrations have been shown to correlate positively with the trend to develop atherosclerosis (Havel, 1995). Atherosclerosis is a progressive disease and the principal cause of cardiovascular diseases in the western world. Cardiovascular diseases are the leading cause of death and disability in industrialized societies (Cheng et al., 2002; Hajjar, 1995). During the progression of atherosclerosis, cholesterol (along with lipids, cellular debris and fibrin) accumulates in the walls of arteries forming plaques that obstruct the flow of blood. This process eventually leads to an increased arterial blood pressure, myocardium infarct and strokes (Hajjar, 1995). Since apoB is required for the assembly of many atherogenic lipoproteins, it is important to understand its biosynthetic process in order to design strategies that will reduce the circulating concentration of apoB, thereby reducing the levels of cholesterol and triglycerides in the blood.

ApoB is a 512 kDa secreted glycosylated, phosphorylated and palmitoylated protein that interacts with several molecular chaperones and various components of the lipoprotein particle assembly machinery (Havel, 1995; Linnik and Herscovitz, 1998). Despite the fact that apoB was shown to be

palmitoylated (Hoeg et al., 1988; Huang et al., 1988; Kamanna and Lee, 1989; Lee, 1991; Lee and Singh, 1990; Zhao et al., 2000), the involvement of palmitoylation in the structure and/or function of this apolipoprotein has not been fully investigated. By using deletion analysis combined with metabolic labeling of truncated human apoB forms secreted from rat hepatoma cells, our laboratory showed that apoB-29, a functional truncated form of apoB that comprises the amino-terminal 29% of the protein, is palmitoylated on its only free cysteine residue (Cys 1085) (Zhao et al., 2000). Our laboratory also showed that palmitoylation at this cysteine residue 1085 is involved in the assembly of the hydrophobic neutral lipid core of the lipoprotein particle as well as in its intracellular localization (Zhao et al., 2000). Although cells do not naturally synthesize apoB-29, this work proved that this truncated form of apoB constitutes a functional model to study the role of palmitoylation in the early biogenesis of apoB-containing lipoprotein particles.

Since very little is known on the role of palmitoylation of secreted proteins, we wanted to further investigate the role of palmitoylation in the assembly and secretion of apoB. Herein, we show that palmitoylation of apoB occurs early in its biogenesis, stimulates ER-Golgi transport rate of apoB-29, doubles apoB-29 secretion efficiency and appears to be involved as a quality control step favoring dissociation of apoB-29 from the ER chaperone CLX. In addition, non-palmitoylated apoB-29 co-localized extensively with the constitutively secreted protein transferrin while palmitoylated apoB-29 showed only partial co-localization and appeared to be enriched in extensions of the ER. Thereby, our

results suggest an important role for palmitoylation in quality control, assembly and secretion of apolipoprotein B containing lipoprotein particles.

3.2 Results

3.2.1 Secretion efficiency of wild type and (Cys1085Ser) apoB-29

In order to study the role of palmitoylation in apoB biogenesis, we compared various quality control aspects of protein secretion using palmitoylated and non-palmitoylated apoB-29 variants. We therefore measured the apoB-29 secretion rate and efficiency, the rate of ER-Golgi transport of apoB-29 (acquisition of endoH resistance assay) and the interactions of apoB-29 with the molecular chaperone CLX.

To do so, McArdle-RH7777 cells stably expressing wild type apoB-29 or (Cys1085Ser) apoB-29 (Zhao et al., 2000) were labeled with [^{35}S]-methionine/cysteine for 15 min (pulse) and the residual intracellular and secreted labeled apoB-29 were collected by immunoprecipitation and quantified by phosphorimaging (Fig.3.1). While the rate of disappearance of both residual apoB-29 inside the cells was very similar, there was a two-fold decrease in the (Cys1085Ser) apoB-29 secretion efficiency compared with that of the wild type apoB-29 (26 ± 0.4% and 54± 1.2% respectively, n=4). This decrease in (Cys1085Ser) apoB-29 secretion efficiency suggests that the non-palmitoylated form of the protein is more actively degraded than the wild type, perhaps due to a longer residence time in the ER.

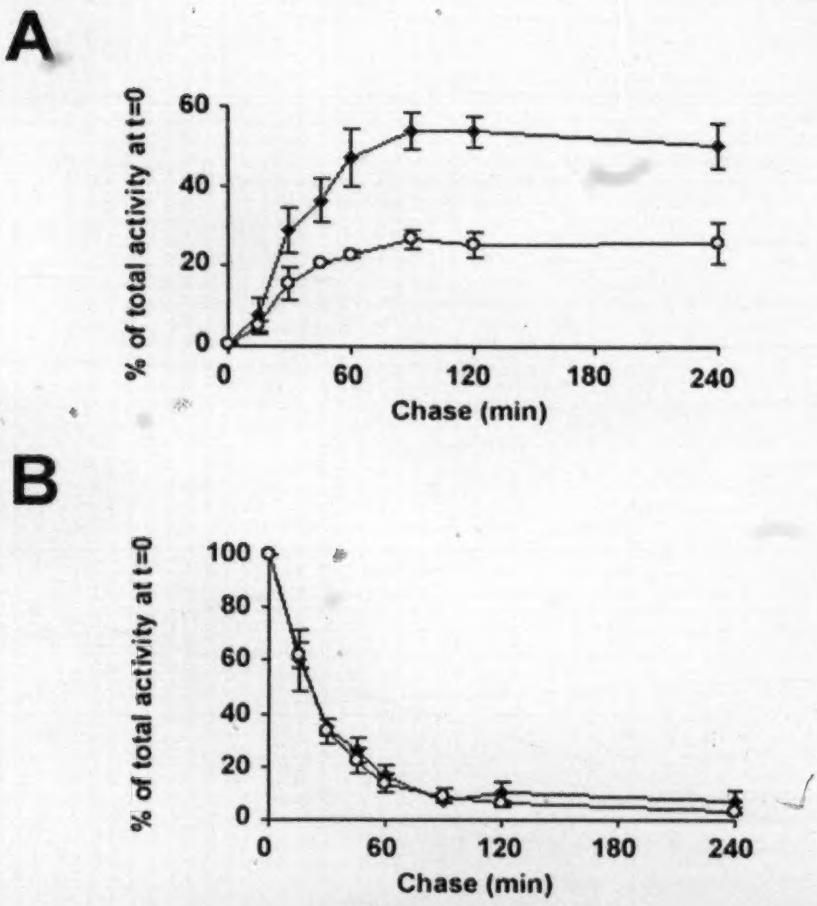


Figure 3.1. **Human Cys1085Ser apoB-29 is secreted less efficiently than wild type apoB-29 from McArdle RH-7777 cells.** [^{35}S]-cys/met pulse-chase analysis of McArdle RH-7777 rat hepatoma cells stably expressing either human wild type apoB-29 or (Cys1085Ser) apoB-29 performed as described in materials and methods. At the indicated times, apoB-29 from the medium (A) as well as from the cells (B) was recovered by immunoprecipitation, solubilized in electrophoresis sample buffer, boiled and separated by Tris-Tricine SDS-PAGE. The specific activity of samples collected at each time point was calculated. The results are expressed as percentage of total activity at t=0 ± S.E. (n=4) as a function of time. Open symbols (Cys1085Ser) apoB-29, closed symbols wild type apoB-29.

3.2.2 Palmitoylation of apoB-29 modulates its ER to Golgi transport

In order to investigate whether the non-palmitoylated form of apoB-29 is more actively degraded than the wild type due to a longer residence time in the ER, we sought to measure differences between the rates of ER to Golgi transport of both wild type and (Cys1085Ser) apoB-29. To do so, we established the kinetics of acquisition of endo H resistance using [^{35}S]-methionine/cysteine metabolically labeled McArdle-RH7777 cells stably expressing both wild type and (Cys1085Ser) apoB-29. While in the ER, proteins are modified with a high mannose oligosaccharide moiety. Once the proteins are transported to the Golgi apparatus, the high mannose moiety is trimmed, fucosylated and complex oligosaccharides are added to yield what is known as a hybrid oligosaccharide (Lodish, 1996). Both ER and medial Golgi forms of the protein are of similar apparent molecular weight. To differentiate the form of a protein that is present in the ER from the one in the Golgi apparatus, proteins were harvested and subjected to endoH cleavage. While endoH is able to trim the high mannose moiety from a protein that is trafficking through the ER, it is unable to trim the fucosylated hybrid oligosaccharides that modify proteins moving along the Golgi apparatus. After the endoH cleavage reaction has been performed, these endoH-sensitive (faster electrophoretic mobility, originating from the ER) and endo H–resistant (slower electrophoretic mobility, originating from the Golgi) forms of a protein can be distinguished by SDS-PAGE and their proportions quantified by densitometry or phosphorimager analysis.

Figure. 3.2 A shows that, at time 0, both wild type apoB-29 and (Cys1085Ser) apoB-29 [^{35}S]-labeled proteins appear uniquely in their cleaved endoH sensitive (lower apparent molecular weight) ER form. At 15 min of chase, higher apparent molecular weight, endo H-resistant, apoB-29 forms start to appear. At 30 min of chase, approximately 60 % of the wild type ^{35}S-apoB-29 and 40% of ^{35}S-(Cys1085Ser) apoB-29 appear in the higher apparent molecular weight (endo H resistant form) and as such are believed to have reached the Golgi. This indicates that the time required to transport 50% of apoB-29 from ER to Golgi seems to be shorter than 30 min for the WT and significantly longer for the non-palmitoylated (Cys1085Ser) apoB-29 expressing cells. Figure 3.2 B represents the quantification of the endo H-sensitive apoB-29 expressed as the percentage of the total amount of signal at each time point, normalized by total amount of proteins before immuno-precipitation, for 3 independent experiments. This plot confirms that the apparent half-life ($t_{1/2}$) (the time at which 50% of the apoB-29 reaches the Golgi) for WT apoB-29 is shorter than that for (Cys1085Ser) apoB-29. From a logarithmic replot of these data, we calculated a $t_{1/2}$ for WT apoB29 of 26 ± 4.4 min and a $t_{1/2}$ for (Cys1085Ser) apoB-29 of 46 ± 1.9 min. Therefore palmitoylated apoB-29 was transported from the ER to the Golgi apparatus almost 2 times faster than non-palmitoylated (Cys1085Ser) apoB-29.

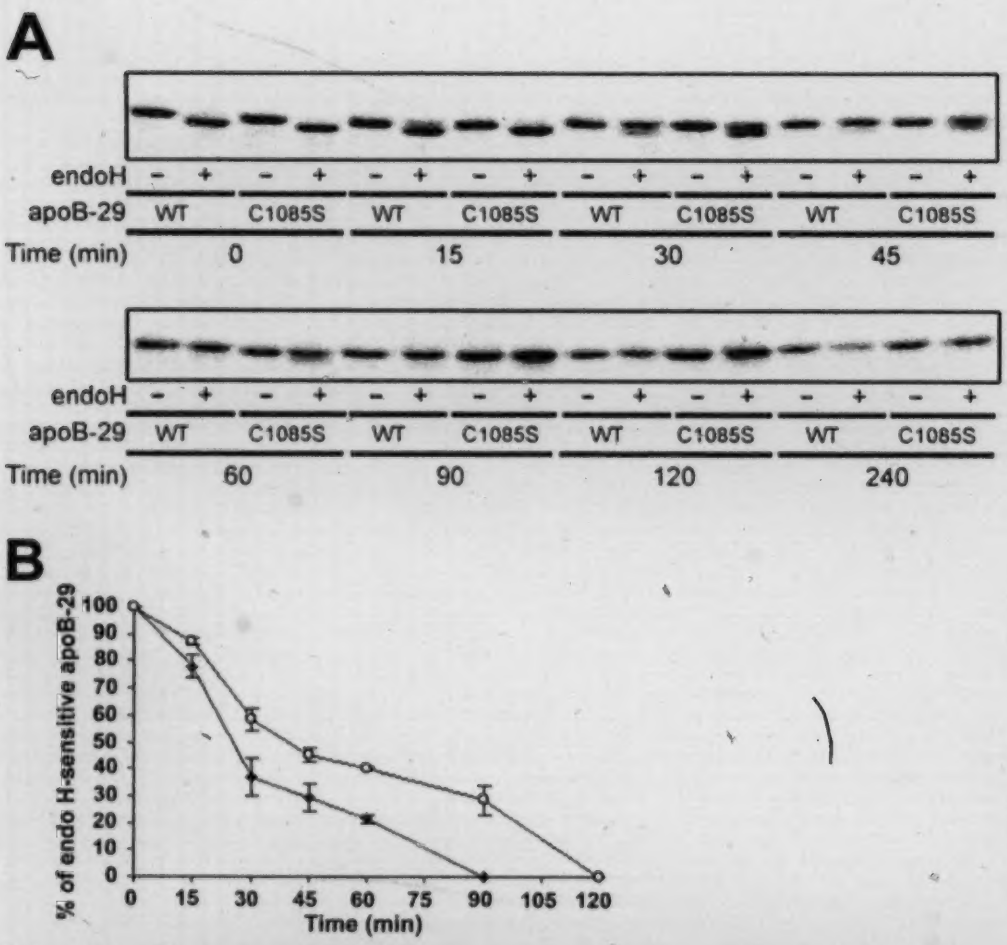


Figure 3.2. **Human (Cys1085Ser) apoB-29 is retained longer in the ER than wild type apoB-29.** ^{35}S-labeled apoB-29 from McArdle RH-7777 cells stably expressing either human wild type or (Cys1085Ser) apoB-29 was recovered during a pulse-chase experiment by immunoprecipitation at different times as described in Fig.3.1. ApoB-29 from cells and media were combined and incubated overnight in the presence or absence of endoH. (A), the digestions were separated by Tris-Tricine SDS-PAGE and the radioactivity incorporated into apoB-29 was detected using phosphorimager analysis. (B), endoH sensitive and resistant bands were quantified using the ImageQuant program and their ratios are plotted as percentage of endoH-sensitive apoB-29 ± S.E. at each time point. The experiments were done in duplicate and repeated three times. Open symbols (Cys1085Ser) apoB-29, closed symbols wild type apoB-29.

3.2.3 Lack of palmitoylation does not affect the overall folding of apoB-29

In order to test whether the ER exit rate differences might be due to differences in protein folding, we assessed the overall folding state of both wild type and (Cys1085Ser) apoB-29 using partial proteolytic cleavage kinetics. To do so, the lumenal content of microsomes prepared from McArdle-RH7777 cells stably expressing either wild type or (Cys1085Ser) apoB-29 were subjected to kinetic partial proteolytic digestion with trypsin. The rationale behind this experiment is that if both proteins are folded in a similar fashion, they will expose the same proteolytic sites. Therefore, they should be degraded at the same rate and display similar proteolytic cleavage intermediates. Fig. 3.3 A demonstrates the high level of similarity of the proteolytic patterns as assessed by the number and size of cleavage intermediates and the speed at which these intermediates appear or disappear (Fig. 3.3 B). These results suggest that, in the lumenal fraction, both wild type and (Cys1085Ser) apoB-29 proteins appear to be folded in a highly similar fashion.

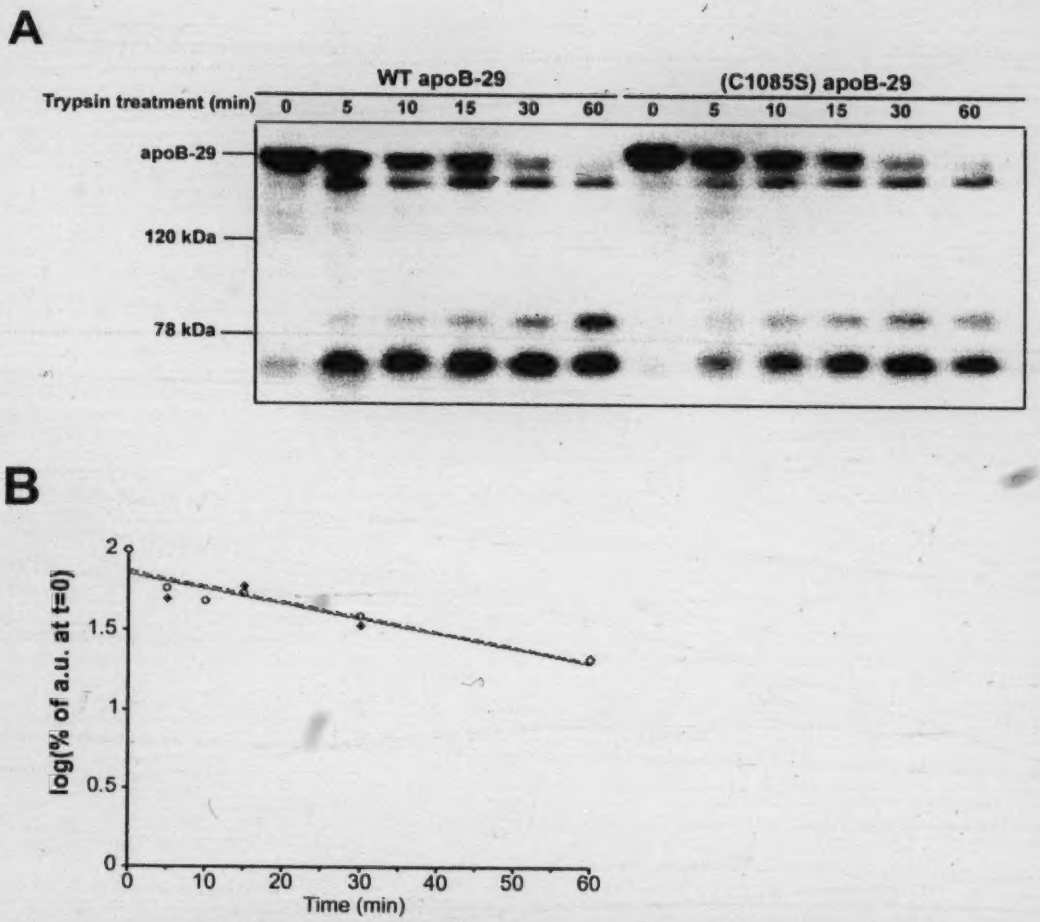


Figure 3.3. **Both wild type and Cys1085Ser human apoB-29 are apparently folded in a similar fashion.** Microsomes obtained from McArdle RH-7777 cells stably expressing either human wild type or (Cys1085Ser) apoB-29 were permeabilized with 0.1 M Na_2CO_3 pH 11.0 and centrifuged to yield an insoluble pellet and a soluble supernatant enriched in lumenal proteins. (A) 1 mg lumenal/supernatant aliquots were subjected to kinetic partial trypsin digestion and, at the indicated times, 0.1 mg aliquots were taken, separated by Tris-Tricine SDS-PAGE and immunoblotted using anti-human apoB antibody. (B) The bands corresponding to full-length apoB-29 were quantified by densitometry and plotted as the logarithm of the percentage of arbitrary units (a.u.) at t=0. Experiments were done in duplicate and repeated three times, most representative results are shown. Open symbols (Cys1085Ser) apoB-29, closed symbols wild type apoB-29.

3.2.4 Calnexin interacts longer with (Cys1085Ser) apoB-29 than apoB-29

To investigate the effect of palmitoylation in the sorting dynamics of apoB-29, we quantified the association of both wild type and (Cys1085Ser) apoB-29 with CLX using cross-linking followed by co-immunoprecipitation as described by others (Berriot-Varoqueaux et al., 2000; Ellgaard and Helenius, 2001; Linnik and Herscovitz, 1998). The data from Fig. 3.4 A and B suggest that loss of palmitoylation at cysteine residue 1085 of apoB-29 results in an increased association with chaperone CLX ($p<0.05$). This increased interaction could likely explain the increased ER retention time and slower ER-Golgi transport rate of (Cys1085Ser) apoB-29 seen in Fig. 3.2 in comparison to WT apoB-29. Thus, it appears that palmitoylation of apoB-29 might be involved in the dissociation of apoB-29-containing lipoprotein particles from CLX in the ER.

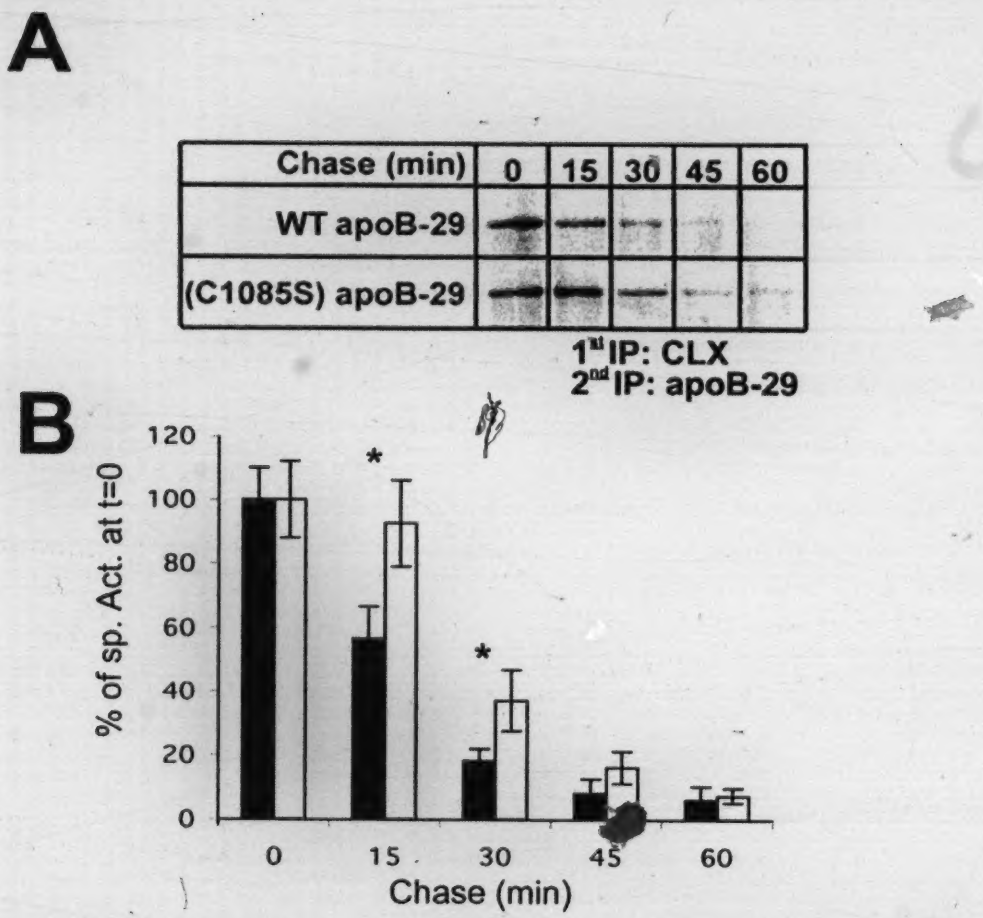


Figure 3.4. **Association of wild type and (Cys1085Ser) apoB-29 with CLX.** McArdle RH-7777 rat hepatoma cells stably expressing either human wild type apoB-29 or (Cys1085Ser) apoB-29 were pulse-labeled for 15 min with [^{35}S]-Cys/Met medium and chased for 1 h. At the indicated times, cells were cross-linked *in situ* and then lysed. CLX was immunoprecipitated and apoB-29 was recovered from the immune complexes using 1D1 antibodies, solubilized in electrophoresis buffer, boiled, separated by Tris-Tricine SDS-PAGE, transferred to a PVDF membrane and CLX as well as apoB-29 were identified by western blot (not shown). The membranes were then dried and the radioactivity incorporated into the bands corresponding to apoB-29 was detected using phosphorimager analysis (A). The specific activity of samples collected at each time point was calculated and the results expressed as percentage of total activity at t=0 ± S.E. (B). Closed bars: wild type apoB-29, open bars: (Cys1085Ser) apoB-29. Experiments were performed three times, asterisks symbolize significant differences between WT and (Cys1085Ser) apoB-29 at the indicated times with $p < 0.05$.

3.2.5 Relative distribution of palmitoylated and non palmitoylated apoB-29 in the main secretory compartments

Using indirect immunofluorescence, palmitoylated and non-palmitoylated apoB-29 showed apparent acylation-dependent differences in sub-cellular localization (Zhao et al., 2000). In order to investigate whether palmitoylation affects the relative distribution of apoB-29 between the ER and Golgi apparatus, microsomes from cells stably expressing either wild type or (Cys1085Ser) apoB-29 were separated on a discontinuous sucrose gradient. The presence of apoB-29 along the gradient was analyzed (Fig. 3.5) and showed that the lack of palmitoylation does not affect the proportions of apoB-29 accumulated along the main compartments of the secretory pathway. Therefore, it appears that palmitoylation only affected the localization of apoB-29 within the ER itself as suggested in Zhao et al. (Zhao et al., 2000) or that our cellular fractionation method is unable to successfully separate the compartments containing both forms of apoB-29 on the basis of density.

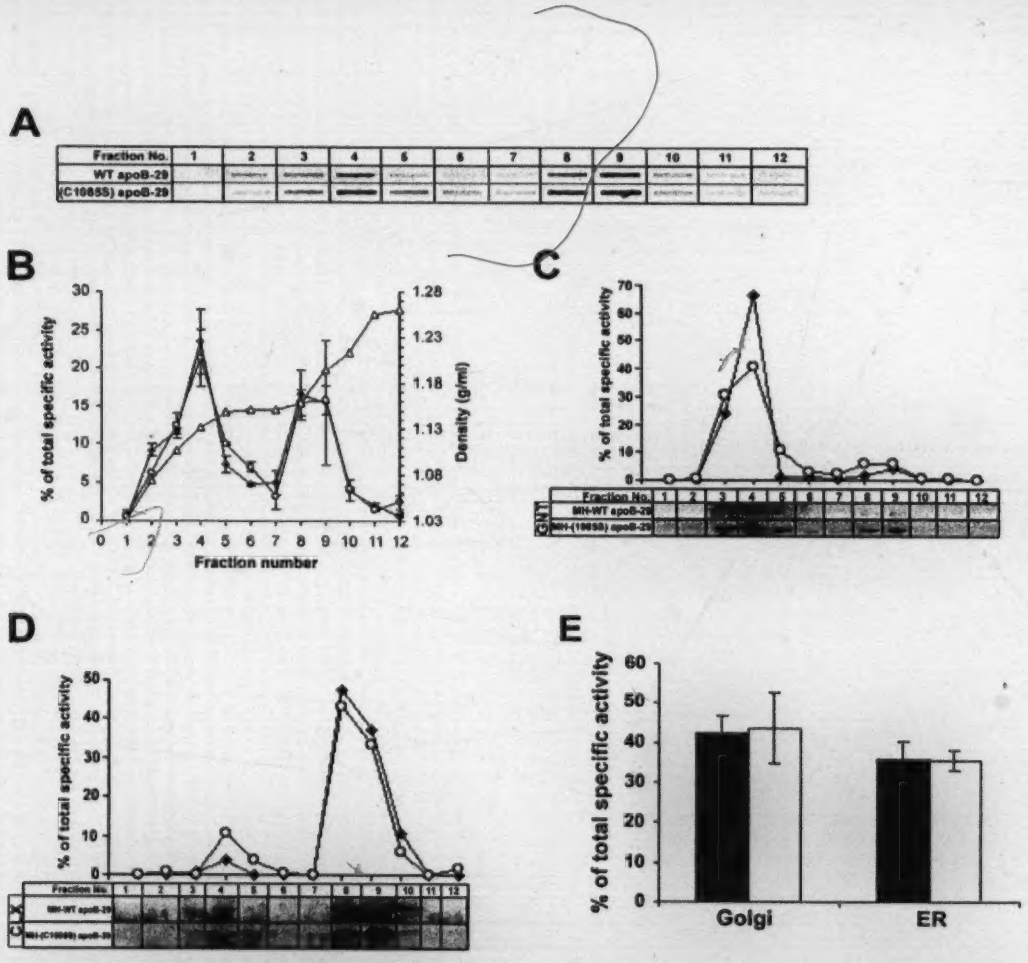


Figure 3.5. **Lack of palmitoylation does not alter the relative distribution of apoB-29 between the ER and Golgi compartments.** Microsomes prepared from McArdle RH-7777 cells stably expressing either wild type (MH-WT apoB-29) or (Cys1085Ser) human apoB-29 (MH-(C1085S) apoB-29) were separated on a discontinuous sucrose gradient as described in materials and methods. (A) Aliquots from fractions were slot-blotted onto nitrocellulose and apoB-29 was immuno-detected using 1D1 anti-human apoB. (B) The slot-blots were quantified by densitometry, normalized by the amount of total protein present in each sample and plotted as % of total specific activity ± S.E. Golgi-containing fractions or ER-containing fractions were determined by the presence of established organelle markers giantin (Golgi) (C) and calnexin (ER) (D) respectively using immunoblotting. (E) ApoB-29 specific activities from Golgi- and ER-containing fractions were added and expressed as % of total specific activity ± S.E. as a function of fraction number. Experiments were done in duplicate and repeated twice. Open symbols/ bars, (Cys1085Ser) apoB-29; closed symbols/ bars, wild type apoB-29; open triangles, fraction density. GNT, giantin; CLX, calnexin.

3.2.6 Intracellular localization of various apoB constructs

We compared the localization of various apoB constructs to that of the constitutively secreted protein transferrin using indirect immunofluorescence and confocal microscopy (Fig. 3.6). In order to investigate the effect of amino acids adjacent to the palmitoylation site on subcellular localization, a further truncated, non-palmitoylated apoB mutant (apoB-18) lacking the last 523 amino acids of apoB-29 was also used (Zhao et al., 2000). While wild type apoB-29 was localized in large vesicular structures distributed throughout the cytosol, non-palmitoylated (Cys1085Ser) apoB-29 and apoB-18 were not. The merged images in Fig. 3.6 show that WT apoB-29 displays a low degree of colocalization with transferrin while both (Cys1085Ser)apoB-29 and apoB-18 colocalize to a high degree with transferrin in large perinuclear structures (identified as the Golgi apparatus). These results indicate that non-palmitoylated apoB-29 and apoB-18 appear to be secreted in a constitutive fashion similar to transferrin. Thus, these non-palmitoylated apoB constructs appear to lack the concentration step at the ER extensions prior to secretion. Therefore, it appears that palmitoylation might confer a signal that is responsible for increasing the partition of apoB-29 to these intra-ER sites.

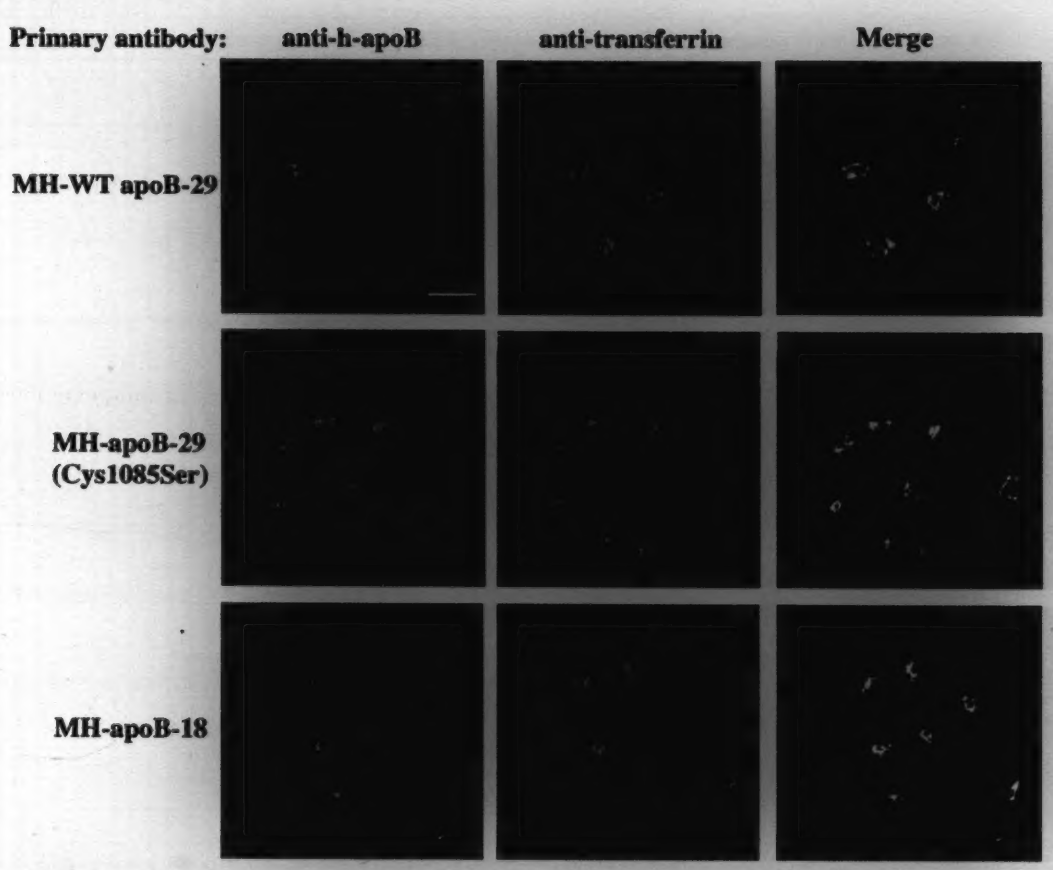


Figure 3.6. **Lack of palmitoylation alters the apparent intracellular localization of human apoB-29.** Colocalization of transferrin with human wild type apoB-29, human (Cys1085Ser) apoB-29 and human apoB 18 in McArdle RH-7777 rat hepatoma cells. Wild type human apoB-29 (MH-WT apoB-29), (Cys1085Ser) human apoB-29 (MH-apoB-29 (Cys1085Ser)) or human apoB18 (MH-apoB-18) were detected using 1D1 anti-human apoB monoclonal antibody and goat anti-mouse IgG conjugated with Texas Red (red, left column). Transferrin (green) was detected with a rabbit polyclonal anti-rat transferrin and goat anti-rabbit IgG conjugated with FITC (green, middle column). The merged images are presented in the right column. Yellow color indicates apparent colocalization of red and green signals. Bar, 10 μm.

3.2.7 Acylation of apoB-29 occurs early during its secretion

In order to identify the apoB acylation site, we metabolically labeled McArdle-RH7777 cells stably expressing wild type apoB-29 with [^{125}I]iodopalmitate for 15 min (pulse), and studied the dynamics of apoB-29 covalent acylation during the chase period by performing acquisition of endoH cleavage resistance assays. Figure 3.7 shows that apoB-29 is palmitoylated in both endoH-sensitive (ER) and endoH-resistant (Golgi) bands at times 0 and 15 min. This suggests that not only wild type apoB-29 is palmitoylated early in its biosynthesis in the ER, but also that this process is likely dynamic and also occurs during the transit of the protein though the Golgi apparatus. This result links the site of palmitoylation of apoB-29 to the site of interaction with chaperones/glycoprotein folding sensors, and, is therefore consistent with a role for protein palmitoylation in quality control and secretion of apoB.

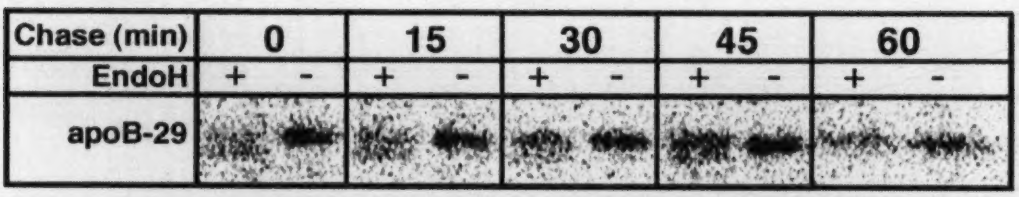


Figure 3.7. **Palmitoylation of Cys 1085 residue in apoB-29 occurs at an early synthesis stage.** McArdle RH-7777 cells stably expressing wild type apoB-29 were metabolically labeled with [^{125}I]iodopalmitate and wild type apoB-29 was recovered at the indicated times by immunoprecipitation as described in materials and methods. ApoB-29 was eluted from the immunocomplexes as described in materials and methods, combined with endoH reaction buffer and incubated overnight in the presence or absence of endoH. Samples were separated by Tris-Tricine SDS-PAGE, blotted onto a PVDF membrane and exposed for 30 days on a radiographic film.

3.3 Discussion

Previous studies demonstrated that lack of palmitoylation of apoB-29 resulted in changes in subcellular distribution under steady state and a five fold reduction in triacylglycerol and cholesteroyl esters content in lipoprotein particles containing mutated apoB-29 in comparison to those containing WT apoB-29 (Zhao et al., 2000). We now show that palmitoylation of apoB occurs in the ER compartment, increases the ER to Golgi transfer rate of apoB-29 as well as apoB-29 secretion efficiency and that this is likely achieved at the molecular level by decreasing the interaction time of the palmitoylated apoB-29 protein with ER chaperone CLX. The longer interaction of the non-palmitoylated form of apoB-29 with CLX might explain the increased degradation as well as the increased ER residence time of this protein. Our data therefore demonstrate the involvement of protein palmitoylation as an active participant in the quality control of a secreted protein, apoB, and suggest that it may also act as a positive transport signal along the secretory pathway.

Since the increase in the retention time of non-palmitoylated apoB-29 in the ER was by no means due to aggregation, nor due to a severe difference in folding, palmitoylation seems to be involved in the quality control of the nascent apoB–containing lipoprotein particle assembly prior to its secretion. In addition to apparently stimulate the dissociation of WT apoB-29 from CLX, apoB-29 palmitoylation might also act as a specialized ER transport signal since it also stimulates ER to Golgi transport and favors concentration of WT apoB-29 to extensions of the ER. In agreement with this possibility is the fact that

lipoprotein particles containing non-palmitoylated apoB-29, which have lower neutral lipid content, do not co-localize with palmitoylated endogenous apoB in these ER extensions (Zhao et al., 2000). This suggests that palmitoylation may act as an intra-organelle sorting signal directing the protein towards specific regions of the secretory pathway where the lipid loading machinery resides. Palmitoylation of apoB-29 could thus stimulate lateral diffusion of the protein within the ER. Lending credibility to this possibility is the fact that non-palmitoylated CCR5, a G protein coupled receptor (GPCR), was shown to have a significantly reduced ER lateral diffusion coefficient in comparison to WT CCR5 (Blanpain et al., 2001).

The fact that non-palmitoylated apoB-29 co-localized extensively with transferrin and appeared to be concentrated in the Golgi is likely due to the lower relative amount of Golgi membranes in comparison to ER membranes present in the cells (ER to Golgi membranes ratio of 10:1) (Warren and Mellman, 1999). Interestingly, non-palmitoylated apoB-29 or apoB-18 containing lipoprotein particles appeared to be sorted without the concentration step in ER extensions and secreted in a more constitutive fashion, like transferrin. These results suggest again a key role for palmitoylation in subcellular localization and assembly of apoB. The fact that apoB-18 (which lacks amino acid sequence from 782 to 1305) was distributed in a similar pattern as (Cys1085Ser) apoB-29 further indicates that palmitoylation, but not amino acids, confer subcellular localization proper to apoB-29 and apoB-100.

Vukmirica et al. claimed that there was a lack of evidence that palmitoylation of apoB-48 is required for lipoprotein secretion (Vukmirica et al., 2003). They based their conclusion on the fact that a non-palmitoylated form of apoB-48 showed no significant differences in apparent intracellular localization or buoyant density of apoB-48 containing lipoprotein particles but showed only a minor effect on the order of 10-20% reduction of secretion efficiency (Vukmirica et al., 2003). Paradoxically, Vukmirica et al. also show a linear dependence of the number of palmitoyl-moieties present on apoB-48 and the secretion efficiency of their various palmitoylated apoB-48 (Vukmirica et al., 2003). Interestingly, in that same figure, the percentage of secreted apoB-48s is shown as approximately 54% and 32% for wild type and non-palmitoylated apoB-$48^{C1085\text{-}1635}$ respectively. Therefore, these results are in agreement with the results obtained with our apoB-29 constructs (54 ± 1.2 % *vs.* 26 ± 0.2% for palmitoylated and non-palmitoylated apoB-29 respectively) since in both cases an approximate 40% reduction was observed which we believe to be important and highly significant. One of the more significant effects published by Vukmirica et al. is that there is a 2-3 fold increase in the proportion of non-palmitoylated apoB-48 bound to microsomal membranes in comparison to WT apoB-48 (Vukmirica et al., 2003). This noteworthy result is also consistent with our data demonstrating a significant increase in binding of non-palmitoylated apoB-29 to CLX, an integral membrane protein. Therefore, palmitoylation may be involved in the regulation of interaction/association of apoB with the inner

membrane leaflet of the lumen along the secretory pathway, perhaps by regulating interaction with one or more molecular chaperones.

The timing of apoB-29 palmitoylation is consistent with its role in the folding, sorting, processing and transport of apoB since it occurs early in apoB biosynthesis and also occurs in a dynamic fashion in the Golgi and possibly further along the secretory pathway as well. Also supporting the possibility of the involvement of palmitoylation as a positive transport signal are several other observations published by other groups on proteins present at the cell surface. Some examples are: hemagglutinin (HA) of type A influenza viruses (Portincasa et al., 1992), the cytoplasmic domain of the HIV-1 envelope glycoprotein (gp160) (Rousso et al., 2000), the mannose 6-phosphate receptor (Schweizer et al., 1996), the human transferrin receptor and many GPCRs (Hawtin et al., 2001; Hayashi and Haga, 1997; Horstmeyer et al., 1996). In all these cases, lack of palmitoylation resulted in impaired trafficking as shown by decreased cell surface expression and increased intracellular accumulation. Thus, loss of palmitoylation of these proteins interfered with their maturation and proper localization processes.

Altogether, and, in addition to our findings on apoB palmitoylation, these observations support an important and perhaps ubiquitous role for palmitoylation as a positive transport signal along the secretory pathway (Berthiaume, 2002). Palmitoylation might therefore be responsible for the regulation of spatio-temporal functional properties of several receptors, signalling proteins and secreted proteins such as apoB via a yet unidentified

mechanism. Alternatively, the non-palmitoylated cysteine residue could play an active role, via its free thiol, in ER-retention and binding to ER-localized chaperones (Burch and Herscovitz, 2000; Isidoro et al., 1996). In such models, the location and regulation of the putative palmitoyl acyl transferase or acyl-protein thioesterase activities would be critical for proper control of the acylated protein's movement along the secretory pathway.

Interestingly, it is well established that the availability of neutral lipids regulates the secretory pool of apoB and that in the absence of neutral lipids most apoB is degraded (Davidson and Shelness, 2000; Davis, 1999; Fisher et al., 1997; Ginsberg, 1997; Gordon et al., 1994). Since palmitoyl-CoA is the substrate for enzymes such as palmitoyl-diacylglycerol acyltransferase and acyl-CoA cholesterol acyltransferase involved in neutral lipid synthesis as well as for the palmitoyl acyltransferase (PAT) that palmitoylates apoB, an emerging concerted model could link increased levels of palmitoyl-CoA (above K_m for these enzymes) to increased activity of the enzymes synthesizing neutral lipids and the PAT that palmitoylates apoB. The ensuing increased availability of neutral lipids as well as increased palmitoylation of apoB would stimulate the rate of apoB transport from ER to Golgi, thereby reducing apoB degradation and overall increasing secretion efficiency.

CHAPTER 4

Post-translational myristoylation of caspase-activated p21-activated protein kinase 2 (PAK2) potentiates late apoptotic events

A version of this chapter has been published in Gonzalo L. Vilas, Maria M. Corvi, Greg J. Plummer, Andrea M. Seime, Gareth R. Lambkin and Luc G. Berthiaume. "Posttranslational myristoylation of caspase-activated p21-activated protein kinase 2 (PAK2) potentiates late apoptotic events". *PNAS* (2006) **103** (17), 6542-6547.

4.1 Overview

Apoptosis, or programmed cell death, is a fundamental process in the development of multicellular organisms. Apoptosis enables an organism to eliminate unwanted or defective cells through an organized process of cellular disintegration that has the advantage of not inducing an inflammatory response. This process plays a key role in the control of normal morphogenesis and development of the immune, endocrine and nervous system (Jiang and Wang, 2004). Improper regulation of apoptosis has been associated with disorders such as cancer, viral infection, autoimmune disease, neurodegenerative disorders, stroke, anemia and AIDS (Wyllie, 1997; Wyllie et al., 1999). Although it is initiated by many physiological and pathological stimuli, all apoptotic cells undergo a similar sequence of morphological changes including membrane blebbing, cell shrinkage, nuclear and cytoplasmic condensation, and a final fragmentation into apoptotic bodies (Kerr et al., 1972).

Two distinct but interconnected apoptotic pathways regulate cell death, the extrinsic or receptor-mediated pathway and the intrinsic or mitochondrial pathway (Hengartner, 2000; Leist and Jaattela, 2001). The family of Cysteinyl ASpartyl ProteASEs, or caspases, is known to be responsible for many of the morphological changes observed during both apoptotic pathways (Nicholson, 1999). The receptor-mediated pathway is activated by binding of ligands (e.g. tumor necrosis factor (TNF) or Fas/CD95 ligand) to their respective receptors, which induces their trimerization, recruitment of Fas-associated death domain protein (FADD) and activation of the initiator caspase, caspase-8. Finally,

caspase-8 activation leads to the activation of executioner caspases (e.g. caspases-3,-6,-7) (Hengartner, 2000). The intrinsic or mitochondrial pathway is activated by a variety of extracellular cues and intracellular insults such as DNA damage, serum withdrawal and heat shock. These stresses lead to loss of mitochondrial membrane potential ($\Delta\Psi m$) and the release of proteins, including cytochrome c and apoptosis inducing factor (AIF), from the mitochondrial inter-membrane space. Cytochrome c then binds to the apoptotic peptidase activating factor-1 (Apaf-1) that activates caspase-9 which further promotes cleavage of executioner caspase-3 (Jiang and Wang, 2004).

Once apoptosis is triggered, executioner caspases cleave a number of intracellular proteins (Nicholson, 1999). In some cases, proteins are inactivated as a result of caspase cleavage. Among the targets of caspases are many cytoskeletal proteins (actin, gelsolin, β-catenin, focal adhesion kinase) and proteins involved in signalling pathways, apoptosis regulation and DNA repair. By cleaving these proteins, it is believed that caspases turn off cell survival and initiate cell disassembly.

Interestingly, following Fas activation, caspase-8 cleaved Bid (a Bcl-2 family member) was shown to be effectively myristoylated at an internal glycine residue in a post-translational fashion by N-myristoyl transferase (NMT) (Zha et al., 2000). Typically, N-myristoylation is a co-translational process in which the fourteen-carbon fatty acid myristate is added to the N-terminal glycine residue via an amide bond after the removal of the initiator methionine residue by an amino-methionyl peptidase. The consensus sequence for NMT protein

substrates is Met-Gly-X-X-X-Ser/Thr/Cys-. The glycine at position 2 is essential for myristoylation, serine, threonine or cysteine is preferred at position 6, and lysine or arginine is preferred at position 7 and/or 8 (Farazi et al., 2001; Resh, 2004). Typically, by itself, a myristoyl moiety added to a protein is not sufficient to confer stable membrane anchoring. Indeed, a second signal in the form of a polybasic stretch of amino acids (as in c-Src) or one or two palmitoyl-cysteine residues (as in Lck NRTK) is often required for stable membrane binding and proper membrane targeting (McCabe and Berthiaume, 1999; Resh, 2004).

Since the original demonstration of post-translational myristoylation (Zha et al., 2000), the N-termini of the C-terminal cleavage products of actin and gelsolin, were also shown to be post-translationally N-myristoylated using recombinant systems (Utsumi et al., 2003). Actin and gelsolin are two proteins involved in cytoskeleton structure and dynamics (Rao and Li, 2004; Silacci et al., 2004). While post-translationally N-myristoylated exogenously expressed tActin-FLAG was shown to co-localize with mitochondria, like myristoylated-tBid, its role during apoptosis is unknown. However, the role of myristoylation in sub-cellular localization of tGelsolin has recently been investigated. Immunofluorescence staining and subcellular fractionation experiments revealed that exogenously expressed myristoylated-tGelsolin did not localize to mitochondria but rather was diffusely distributed in the cytoplasm and appeared to have anti-apoptotic activity (Sakurai and Utsumi, 2006).

Amino acid sequence analysis adjacent to a known caspase-cleavage site (Nicholson, 1999) of p21-activated kinase-2 (PAK2) revealed a putative internal

myristoylation sequence (**Gly**-Ala-Ala-Lys-**Ser**-Leu-Asp-Lys-Gln-Lys-Lys-Lys) (Bokoch, 2003; Farazi et al., 2001; Resh, 2004). Furthermore, this analysis also revealed that the putative myristoylatable glycine is followed by a short stretch of basic amino acid residues often required for membrane association (Farazi et al., 2001; McCabe and Berthiaume, 1999; Resh, 2004). PAK2 is a serine/threonine kinase activated by small GTPases CDC42 and Rac (Knaus et al., 1995) and is involved in the regulation of various cytoskeletal functions including cell motility (Bokoch, 2003) and membrane blebbing during apoptosis (Rudel and Bokoch, 1997). Of the six PAK family members, only PAK2 is ubiquitously expressed and cleaved by caspase-3 (Bokoch, 2003; Jakobi et al., 2003; Rudel and Bokoch, 1997). This cleavage removes most of the N-terminal regulatory domain and generates a constitutively active C-t-PAK2, a 34 kDa C-terminal fragment that contains the entire catalytic domain (Rudel and Bokoch, 1997).

In this study, we demonstrate that upon caspase-3 cleavage of PAK2, the 34 kDa C-terminal fragment (C-t-PAK2) is indeed post-translationally myristoylated. We show that myristoylation directs C-t-PAK2 to specific regions of the plasma membrane (membrane ruffles) and internal membranes where it might play a role during the onset of late apoptotic events. We present data demonstrating that myristoylation of C-t-PAK2 significantly promoted cell death via an increased signalling through the stress activated signalling pathway leading to JNK phosphorylation. Surprisingly, myristoylation of C-t-PAK2 promoted the later events of cellular death without compromising mitochondrial

integrity. Our results indicate that C-t-PAK2 is a prototypical post-translationally myristoylated caspase-cleaved protein that is part of a novel mechanism of cellular death regulation. To further illustrate this possibility, we also demonstrate the existence of at least 9 post-translationally myristoylated proteins in Jurkat cells undergoing apoptosis.

4.2 Results

4.2.1 Identification of a myristoylation consensus sequence downstream the caspase-3 cleavage site in PAK2

PAK2 was shown to be proteolytically cleaved by caspase-3 at position Asp^{212} to yield a 28 kDa amino-terminal fragment containing the regulatory domain and a 34 kDa carboxy-terminal fragment containing the kinase domain (C-t-PAK2) of the protein (Bokoch, 2003; Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997; Rudel and Bokoch, 1997; Rudel et al., 1998). Interestingly, the new amino-terminal end of the 34 kDa C-t-PAK2 fragment contains the classical Gly-X-X-X-Ser myristoylation consensus sequence followed by a polybasic stretch of amino acids often required for proper membrane anchoring (Resh, 2004) (Fig. 4.1 A). In order to determine whether C-t-PAK2 was myristoylated, three peptides encompassing the first 10 amino acids of C-t-PAK2 were synthesized to be used in an in vitro enzymatic assay followed by mass spectrometry (MS) analysis. The first peptide contained the wild type sequence GAAKSLDKGK (Gly^{213}-N10-PAK2), the second sequence AAAKSLDKGK corresponded to a non-myristoylatable polypeptide where the essential glycine (Gly^{213}) has been substituted to alanine (Ala^{213}-N10-C-t-PAK2)

and the third one was a mass standard consisting of a chemically myristoylated Gly^{213}-N10-PAK2 (myr-N10-C-t-PAK2). These peptides were incubated in the presence or absence of the highly selective yeast NMT (Boutin, 1997; Farazi et al., 2001) with or without the myristoyl donor myristoyl-Coenzyme A. Following the enzymatic reaction, the mass of the resulting peptides was analyzed by MS (Fig. 4.1 B). When incubated with NMT and myristoyl-Coenzyme A, Gly^{213}-N10-C-t-PAK2 presented two distinctive peaks at 1255 m/z and 1044 m/z corresponding to the myristoylated and non-myristoylated forms of the peptide respectively. On the other hand, Ala^{213}-N10-C-t-PAK2 did not show a peak at 1268 m/z indicating that this peptide was not a substrate for NMT. Taken together, these results suggest that the peptide encompassing the first 10 amino acids of the caspase-3 cleaved carboxy-terminal kinase domain of PAK2 is enzymatically myristoylated *in vitro* by NMT.

In order to further confirm whether C-t-PAK2 was myristoylated in vivo, we metabolically labeled Jurkat T cells with [^{3}H]myristate and treated them with or without staurosporine (STS) (Na et al., 1996) for 5 hours. Fluorography analysis of the cell lysates separated by SDS-PAGE indicated that only the immunoprecipitated endogenous 34 kDa C-t-PAK2 incorporated radiolabeled myristate (Fig. 4.2 A) while the full length endogenous PAK2 did not. This result suggested that caspase-3 cleaved C-t-PAK2 is post-translationally myristoylated *in vivo* in apoptotic Jurkat T cells.

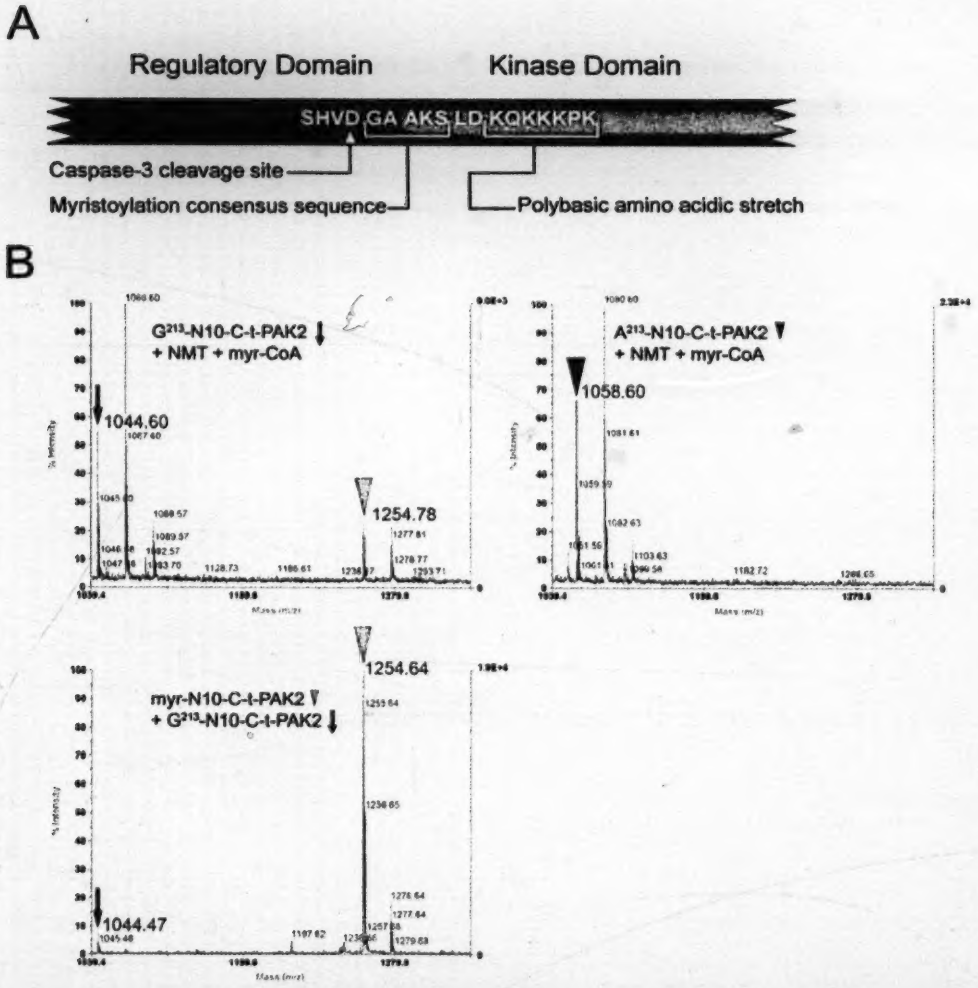


Figure 4.1. **The N-terminal decapeptide of C-t-PAK2 is myristoylated *in vitro*.** (A) A schematic representation of the inter-domain region of PAK2. The diagram shows the cleavage site for caspase-3, the putative myristoylation sequence and a polybasic stretch of amino acids required for stable membrane binding and targeting. (B) Decapeptides encompassing either the first 10 amino acids of C-t-PAK2 (Gly^{213}-N10-C-t-PAK2) or a non-myristoylatable Ala^{213}-N10-C-t-PAK2 were incubated in the presence of NMT and myristoyl-CoA followed by mass spectrometry analysis. Gly^{213}-N10-C-t-PAK2 and a chemically myristoylated Gly^{213}-N10-PAK2 (myr-N10-C-t-PAK2) were used as negative and positive myristoylation controls respectively. Black arrows indicate the position of the non-myristoylated Gly^{213}-N10-C-t-PAK2 peak while red and black arrowheads indicate the position of myristoylated-Gly^{213}-N10-C-t-PAK2 and non-myristoylated Ala^{213}-N10-C-t-PAK2 respectively (experiment performed by Dr. Maria M. Corvi).

4.2.2 Post-translational myristoylation of C-t-PAK2 is catalyzed by NMT

To determine whether post-translational myristoylation of C-t-PAK2 is catalyzed by NMT, we used the NMT inhibitor 2-hydroxymyristic acid (HMA). When incorporated into the cells, HMA is metabolically converted to 2-hydroxymyristoyl-CoA, a specific inhibitor of NMT (Paige et al., 1990) and does not inhibit other protein acylation events such as protein palmitoylation (Galbiati et al., 1996; Nadler et al., 1993). When Jurkat T cells were cultured in the presence or absence of 1 mM HMA, metabolically labeled with [^{3}H]myristate and induced to undergo apoptosis with STS/CHX cocktail or left untreated (Fig. 4.2 B), HMA prevented [^{3}H]myristate incorporation into endogenous C-t-PAK2 but did not impair caspase-3 mediated PAK2 cleavage upon STS treatment. HMA treatment alone did not induce PAK2 cleavage in Jurkat T cells. The specific HMA inhibition further illustrates the involvement of NMT in endogenous C-t-PAK2 myristoylation.

4.2.3 Many proteins undergo post-translational myristoylation during apoptosis

In order to study whether post-translational myristoylation was a process restricted to Bid, actin, gelsolin (Utsumi et al., 2003; Zha et al., 2000) and now PAK2 or was a more widely used modification process during the onset of apoptosis, the metabolically radiolabeled proteins contained in the post-PAK2-immunoprecipitation supernatants from the previous experiment were analyzed

by SDS-PAGE followed by autoradiography. Figure 4.2 C shows that at least 9 proteins exhibit apparent post-translational myristoylation. As post-translational myristoylation criteria, we only considered acylated proteins whose incorporation of tritiated myristate was drastically reduced in the presence of the NMT inhibitor HMA upon induction of apoptosis with STS/CHX cocktail. The identity of these 9 proteins is currently being investigated. These results indicate that there are several cases of post-translational myristoylation of proteins during the onset of apoptosis and that this phenomenon might play an important novel role in the regulation of apoptosis.

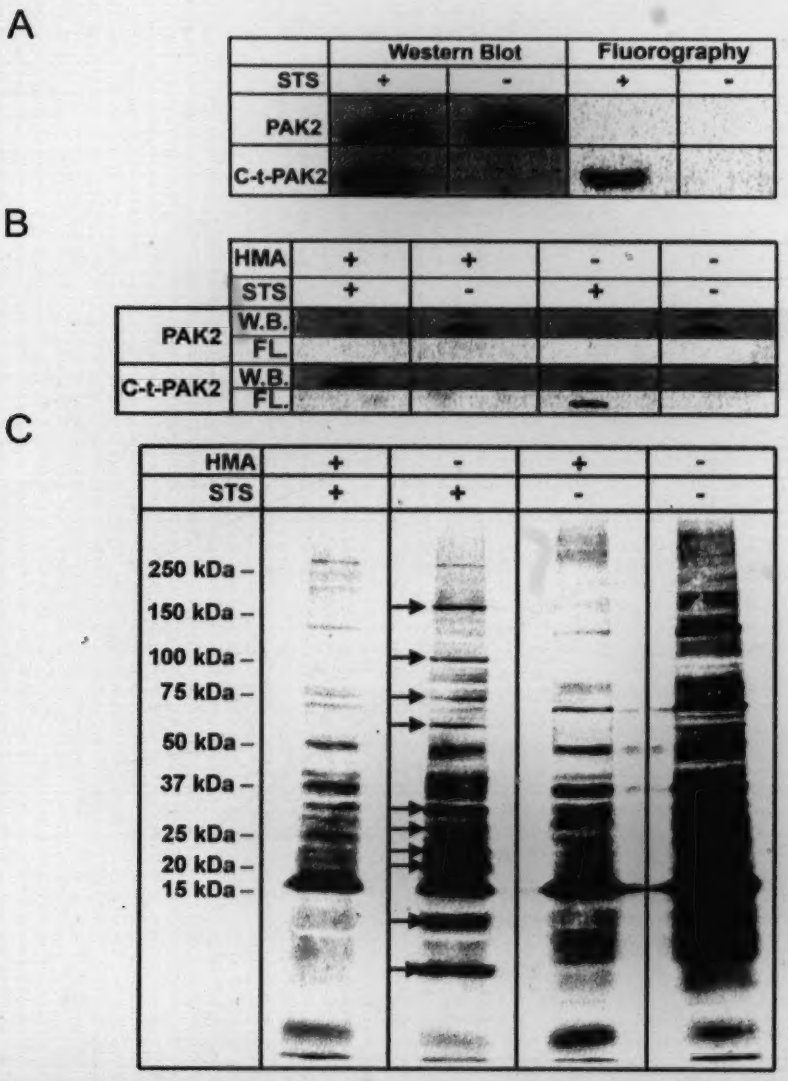


Figure 4.2. **C-t-PAK2 is post-translationally myristoylated on Gly 213 by NMT during apoptosis in Jurkat T cells.** Jurkat T cells metabolically labeled with [9,10(n)-^{3}H]myristate were incubated in the absence or presence of 2.5 μM STS and 5 μg/ml CHX (STS) to induce apoptosis (A) or labeled and induced to undergo apoptosis in the absence or presence of 100 μM 2-hydroxy myristic acid (HMA) for 5 h (B). Cells were lysed and PAK2 was immunoprecipitated. Immunocomplexes were analyzed by western blotting (W.B.) and fluorography (FL.). (C) Post-immunoprecipitation supernatants were TCA- precipitated and analyzed by autoradiogram. Arrows indicate acylated proteins whose incorporation of tritiated myristate was drastically reduced in the presence of the NMT inhibitor HMA upon induction of apoptosis with STS.

4.2.4 The myristoyl moiety is attached to C-t-PAK2 through an amide bond

In order to further confirm whether C-t-PAK2 was *N*- or *S*-acylated, COS-7 cells were transfected with plasmids expressing either a recombinant C-terminally myc-tagged C-t-PAK2 construct (Gly^{213}-C-t-PAK2-myc), a non-myristoylatable A^{213}-C-t-PAK2-myc, a chimeric GAP-43-GFP containing the first 10 amino acids of the dually palmitoylated protein (a protein known to be S-acylated (McCabe and Berthiaume, 1999)) and GFP. After transfection, cells expressing PAK2 constructs were metabolically labeled with [^{3}H]myristate while cells expressing GAP-43-GFP or GFP were labeled with [^{125}I]iodopalmitate as described in McCabe and Berthiaume (1999) and lysed (McCabe and Berthiaume, 1999). Chimeric C-t-PAK2-myc and GFP recombinant proteins were immunoprecipitated, electrotransferred to PVDF membranes and detected by western blot. The membranes were then treated with 0.2 N KOH (pH ~13.0), which cleaves both thioesters and oxyesters or 1 M Tris-HCl, pH 7.0 (Armah and Mensa-Wilmot, 1999; Zhao et al., 2000). Gly^{213}-C-t-PAK2-myc but not Ala^{213}-C-t-PAK2-myc incorporated [^{3}H]myristate. The label incorporated into Gly^{213}-C-t-PAK2-myc was resistant to alkali treatment (0.2 N KOH), which efficiently deacylated GAP-43-GFP. Neutral Tris treatment failed to deacylate neither Gly^{213}-C-t-PAK2-myc nor GAP-43-GFP (Fig. 4.3). Since the substitution of Gly^{213} by Ala in A^{213}-C-t-PAK2-myc did abrogate incorporation of [^{3}H]myristate into the protein, the results showing that label is incorporated in an

alkali resistant fashion indicate that [^{3}H]myristate label is linked to the amino-terminal glycine residue of C-t-PAK2-myc via an amide bond.

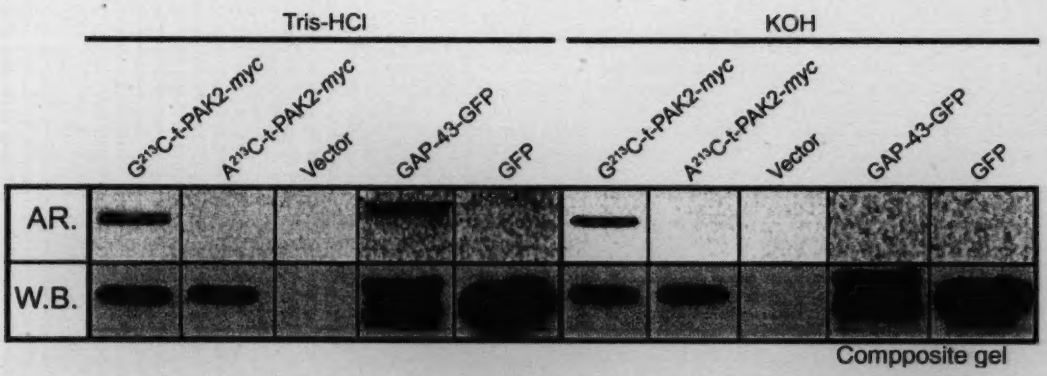


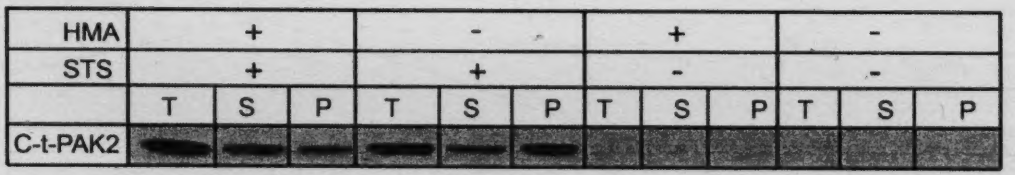
Figure 4.3. **The myristoyl moiety is attached to C-t-PAK2 through an amide bond**. COS-7 cells were transfected with plasmids allowing expression of either Gly^{213}-C-t-PAK2-myc, the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras, vector alone, a palmitoylatable (non-myristoylated) chimeric GAP-43-GFP or GFP. Transfected cells were metabolically labeled with [9, 10(n)-^{3}H]myristate or [^{125}I]iodopalmitate for 4 h and lysed. Expressed proteins were immunoprecipitated and analyzed by western blotting (W.B.). PVDF membranes were then soaked for 24 h in either 0.2 N KOH or 1M Tris-HCl pH 7.0 and subjected to imaging with phosphorimager screens (AR.).

4.2.5 HMA alters the subcellular localization of endogenous C-t-PAK2 during apoptosis

Post-translationally myristoylated Gly^{213} is adjacent to a short polybasic domain, a combination known to lead to membrane anchoring (McCabe and Berthiaume, 2001; Resh, 2004). In order to investigate the role of myristoylation in the subcellular localization of C-t-PAK2, Jurkat T cells were incubated in the absence or presence of HMA, treated with or without STS for 5h and subjected to a subcellular fractionation protocol that yielded a soluble cytosolic fraction (S) and an insoluble fraction containing most cellular membranes (P).

In apoptotic Jurkat T cells, the vast majority of C-t-PAK2 remained soluble in the presence of the NMT inhibitor HMA (Fig. 4.4 A) while C-t-PAK2 was found predominantly in the membrane fractions (P) in the absence of HMA. These results indicate that after caspase-3 cleavage, endogenous C-t-PAK2 is post-translationally myristoylated and associates more efficiently with membranes.

A



B

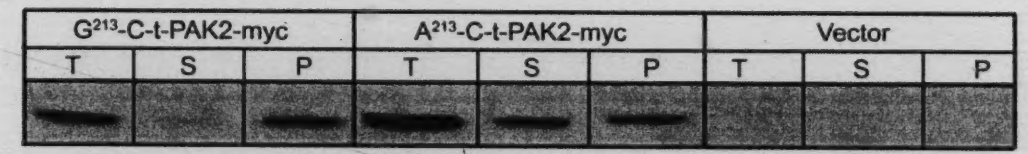


Figure 4.4. **Myristoylation of C-t-PAK2 promotes membrane association.** (A) Jurkat T cells were treated for 5 h with or without 2.5 μM STS and 5 μg/ml CHX (STS) to induce apoptosis in the absence or presence of 100 μM 2-hydroxymyristic acid (HMA). Cells were lysed and fractionated into total (T), cytosolic (S) and membrane (P) fractions and the presence of C-t-PAK2 was analyzed by western blot/ECL. (B) COS-7 cells were transfected with plasmids expressing either Gly^{213}-C-t-PAK2-myc, the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras or vector alone and were analyzed 12-14 h post-transfection. Cells were subjected to subcellular fractionation and C-t-PAK2 detection as in (A).

4.2.6 Myristoylation of C-t-PAK2 promotes membrane association

In order to further clarify the impact of post-translational myristoylation on the subcellular localization of C-t-PAK2, we compared the subcellular partitioning properties of the non-myristoylatable mutant A^{213}-C-t-PAK2-myc to those of Gly^{213}-C-t-PAK2-myc in transfected COS-7 cells at 12-14 h post-transfection. Fig. 4.4 B shows that while the myristoylatable Gly^{213}-C-t-PAK2-myc was found almost exclusively in the membrane-containing fraction (P), the non-myristoylatable A^{213}-C-t-PAK2 was found predominantly in the cytosolic fraction (S). This observation confirmed our previous findings (Fig. 4.4 A) in Jurkat T cells with endogenous C-t-PAK2. In the experiments where myristoylation of C-t-PAK2 was abrogated by chemical inhibition of NMT with HMA or by site-directed mutagenesis, there was some residual C-t-PAK2 in the membrane fraction. Whether this residual membrane associated C-t-PAK2 is due to intrinsic membrane binding properties, impurities in the fractionation protocol or incomplete NMT inhibition by the competitive inhibitor HMA is not known.

Several acylated signalling proteins have been documented to be present in lipid rafts (Oh and Schnitzer, 1999). In order to further characterize the nature of the membranes where C-t-PAK2 was found anchored; we studied the effect of post-translational myristoylation of C-t-PAK2 on lipid rafts association. To do so, COS-7 cells transfected with Gly^{213}-C-t-PAK2-myc or the non-myristoylatable mutant A^{213}-C-t-PAK2-myc were used to prepare lipid rafts-

enriched membranes with the use of a detergent-based extraction technique followed by discontinuous sucrose gradient centrifugation and western blot analysis (McCabe and Berthiaume, 2001).

Unlike the lipid rafts protein marker caveolin, which was mostly enriched in the low-density raft fractions at the 5–30% sucrose interface (fractions 3 and 4), both myristoylatable and non-myristoylatable C-t-PAK2-myc chimeras were present, together with the non-raft marker protein transferrin receptor, in the non-buoyant fractions 11 and 12 suggesting that addition of the myristoyl moiety is not sufficient to localize C-t-PAK2 to lipid rafts (Fig. 4.5).

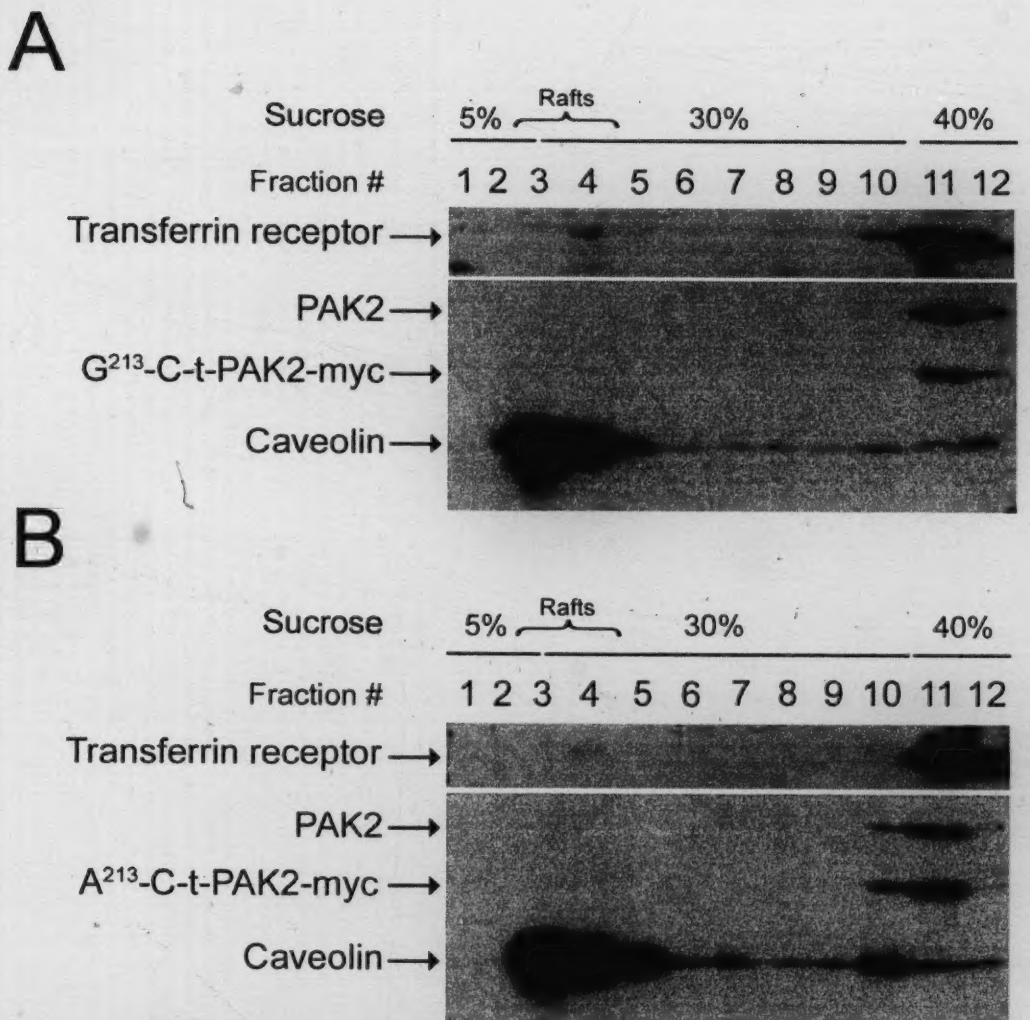


Figure 4.5. **Sub-cellular distribution of C-t-PAK2 into caveolin-enriched lipid rafts.** COS-7 cells were transiently transfected with either Gly^{213}-C-t-PAK2-myc (A) or the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras (B) and were analyzed 12-14 h post-transfection. Cells were homogenized in lipid rafts lysis buffer and the distribution of the C-t-PAK2-myc chimeras as well as endogenous PAK2, caveolin and the non-raft marker transferrin receptor were determined with the use of a discontinuous 5–40% sucrose gradient. Fractions were collected from the top of the gradient, TCA precipitated, separated by SDS-PAGE (12.5%), and analyzed by immunoblotting.

4.2.7 C-t-PAK2 is localized to membrane ruffles and endosomes

The subcellular localization of C-t-PAK2 was further analyzed in transiently transfected COS-7 cells expressing either the myristoylatable or the non-myristoylatable C-t-PAK2-myc chimera at 12-14 h post-transfection. Figures 4.6 to 4.9 show that the Gly^{213}-C-t-PAK2-myc was found primarily at the plasma membrane with apparent enrichment in membrane ruffles as judged by the extent of co-localization with the actin cytoskeletal marker phalloidin. Interestingly, actin staining pattern appeared different in cells expressing Gly^{213}-C-t-PAK2-myc, Ala^{213}-C-t-PAK2-myc or vector alone. Actin-rich membrane ruffles were found predominantly in cells transfected with Gly^{213}-C-t-PAK2-myc. Cells expressing Gly^{213}-C-t-PAK2-myc or Ala^{213}-C-t-PAK2-myc were also typically devoid of stress fibers spanning the cytosol (Fig. 4.8). The non-myristoylatable A^{213}-C-t-PAK2-myc appeared diffuse and completely cytosolic (Fig. 4.6). Contrasting with the nuclear localization of N-terminally tagged GFP-PAK2 constructs reported by Koeppel *et al.* and Jakobi *et al.* (Jakobi et al., 2003; Koeppel et al., 2004); both of our C-t-PAK2-myc chimeras were excluded from the nucleus (Fig. 4.6). These results suggest that myristoylation of C-t-PAK2 is important for proper cellular membrane localization and may stimulate membrane ruffle formation and cytoskeletal reorganization. Additionally, we observed partial co-localization with perinuclear vesicular structures identified as endosomes as judged by the co-localization of Gly^{213}-C-t-PAK2-myc with the early endosomal marker DiI-LDL (Fig. 4.9). The striking difference in localization patterns of myristoylatable and non-myristoylatable forms of C-t-PAK2-myc also

suggests that the myristoylation efficiency of these proteins is likely very high if not complete.

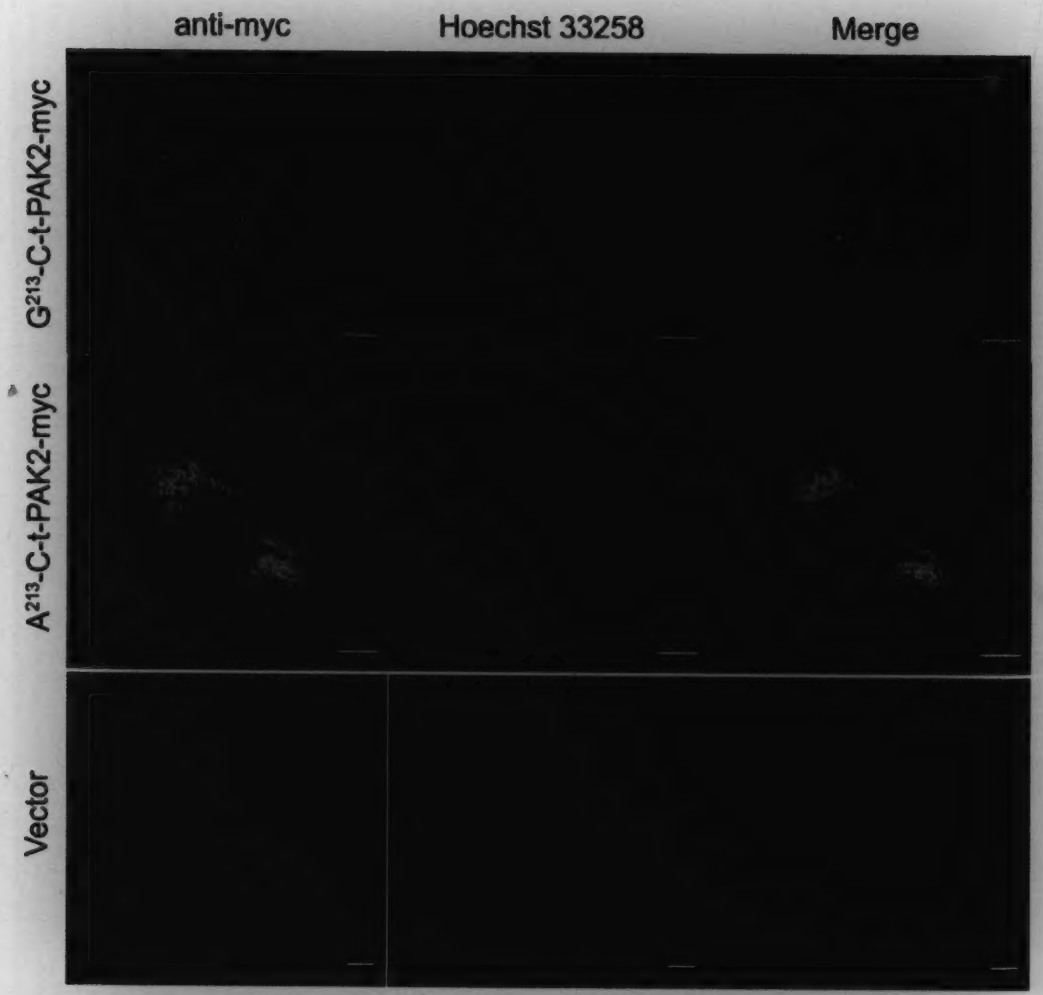


Figure 4.6. **Myristoylation is required for proper intracellular localization of C-t-PAK2-myc.** Indirect immunofluorescence confocal micrographs of COS-7 cells transfected with plasmids allowing expression of either Gly^{213}-C-t-PAK2-myc, the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras or vector alone at 12-14 h post-transfection. Gly^{213}-C-t-PAK2-myc localizes to membranes and membrane ruffles while A^{213}-C-t-PAK2-myc chimera remains mostly cytosolic. Shown are C-t-PAK2-myc chimeras (anti-myc, green) and nuclei (Hoechst 33258 staining, blue). Scale bars represent 10 μm.

Figure 4.7. **Apparent localization of Gly^{213}-C-t-PAK2-myc to membranes and membrane ruffles.** Confocal image depicting a Z-stack reconstruction of a COS-7 cell transfected with Gly^{213}-C-t-PAK2-myc and analyzed as in figure 4.6 showing, in more detail, the apparent membrane ruffle and endosomal localization of the protein. Scale bars represent 10 μm.

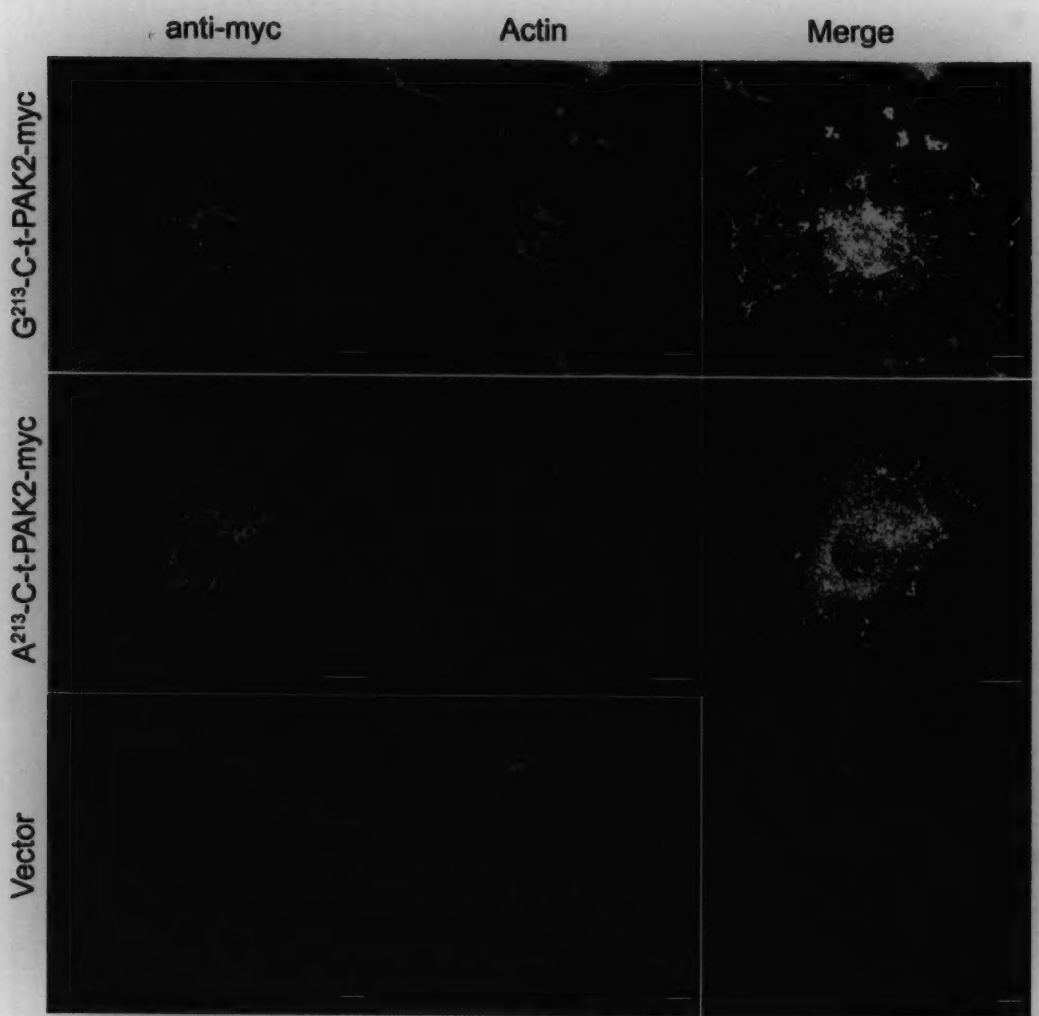


Figure 4.8. **Gly^{213}-C-t-PAK2-myc localizes to membranes and membrane ruffles.** Indirect immunofluorescence confocal micrographs of COS-7 cells transfected with either Gly^{213}-C-t-PAK2-myc, the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras or vector alone at 12-14 h post-transfection, confirming the localization of Gly^{213}-C-t-PAK2-myc to plasma membrane ruffles as indicated by co-localization with actin (red). The merged images are presented in the right hand column. Yellow indicates apparent co-localization of the green (myc) and red signals. Scale bars represent 10 μm.

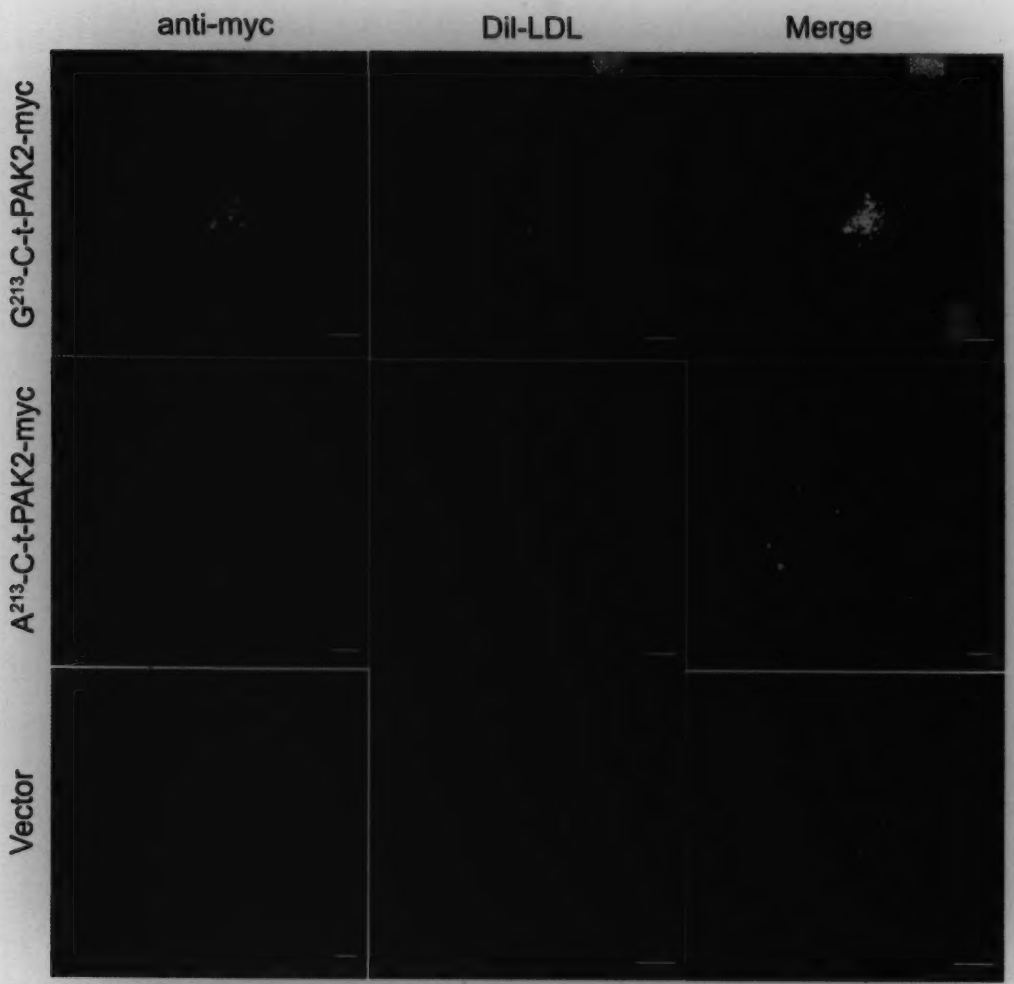


Figure 4.9. **Gly213-C-t-PAK2-myc partially co-localizes with endosomes.** One hour before processing them for indirect immunofluorescence, live COS-7 cells transfected for 12-14 h with plasmids allowing expression of either Gly^{213}-C-t-PAK2-myc, the non-myristoylatable A^{213}-C-t-PAK2-myc chimeras or vector alone were incubated with 1 mg/ml Dil-LDL to allow incorporation of the fluorescent lipoprotein particle into endosomes. After fixation and processing, C-t-PAK2-myc chimeras (green) and endosomes (red) were detected. The merged images are presented in the right hand column. Yellow indicates apparent co-localization of the green and red signals. Scale bars represent 10 μm.

4.2.8 The first 15 amino acids of C-t-PAK2 are sufficient to direct EGFP to membranes

To test whether the combination of myristate and the polybasic domain found at the N-terminus of C-t-PAK2 was sufficient for targeting C-t-PAK2 to membranes, the first 14 amino acids of C-t-PAK2 (G-A-A-K-S-L-D-K-Q-K-K-K-P-K) were appended at the N-terminus of EGFP (Gly213-N15-EGFP). In addition, the non-myristoylatable version Ala^{213}-N15-EGFP was also engineered. In both fixed and live transfected COS-7 cells, the myristoylated Gly^{213}-N15-EGFP was excluded from the nucleus and localized to the plasma membrane, membrane ruffles and perinuclear vesicular structures in a pattern highly similar to that of myristoylated C-t-PAK2-myc (Fig. 4.10). In contrast, the non-myristoylated Ala^{213}-N15-EGFP was found, like EGFP, partly in the cytosol but almost exclusively in the nuclei (Fig. 4.10). These results indicate that the first 14 amino acids of C-t-PAK2 containing the myristoylation consensus sequence and a short polybasic domain are sufficient to localize EGFP to the plasma membrane, membrane ruffles and internal structures. This localization pattern is lost when the myristoylation consensus sequence is abrogated. This confirms that myristoylation is required for proper membrane association and subcellular localization of C-t-PAK2. In addition, the myristoylation efficiency of the Gly^{213}-N15-EGFP construct is likely very high if not complete as judged by its drastic difference in subcellular localization when compared to that of Ala^{213}-N15-EGFP.

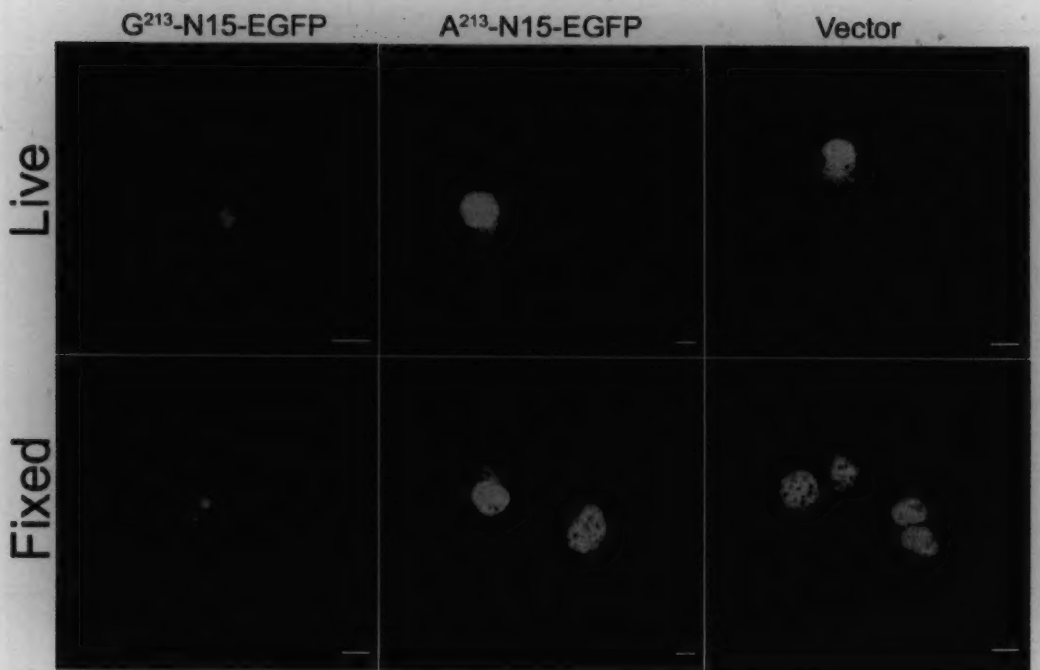


Figure 4.10. **The N-terminal 14 amino acids of C-t-PAK2 encompassing the myristoylation signal and a short polybasic domain are sufficient to localize EGFP to membranes.** COS-7 cells were transfected with plasmids expressing chimeric EGFPs in which the first 14 amino acids of C-t-PAK2 (Gly^{213}-N15-EGFP), the corresponding non-myristoylatable mutant Ala^{213}-N15-EGFP were inserted after the initiator methionine or vector alone. Images obtained 12-14 h post-transfection. EGFP was detected either by live cell fluorescence microscopy (Live) or indirect immunofluorescence (Fixed) by confocal microscopy. Scale bars represent 10 μm.

4.2.9 Myristoylation enhances the capacity of C-t-PAK2 to induce cell death

Caspase-3 mediated cleavage of PAK2 has been observed during apoptosis (Jakobi et al., 2001; Rudel and Bokoch, 1997; Rudel et al., 1998) and overexpression of the N-terminally tagged C-terminal cleavage product C-t-PAK2 has been shown to induce cell death (Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997). However, all these previous studies assessed the apoptotic activity of the non-physiologically relevant non-myristoylated form of C-t-PAK2. In order to study the physiological form of C-t-PAK2 and assess the importance of post-translational myristoylation of C-t-PAK2 on its ability to induce cell death, we compared the capacity of Gly^{213}-C-t-PAK2-myc and A^{213}-C-t-PAK2-myc at inducing cell death using an established co-transfection assay (Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997). Since the expression of C-t-PAK2 leads to nuclear condensation, adherent cell shrinking and rounding up without exhibiting some of the classical hallmarks of programmed cell death (no phosphatidylserine externalization and absence of TUNEL reactivity) (Bisson et al., 2003; Lee et al., 1997) the percentage of cells undergoing programmed cell death at different times after co-transfection was calculated as the percentage of green cells presenting condensed/fragmented nuclei and rounded/retracted/highly refractive morphology as described in Lee *et al.*, Jakobi *et al.* and Koeppel *et al.* (Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997) (Fig. 4.11 A). Expression of the myristoylatable Gly^{213}-C-t-PAK2-myc chimera resulted in 52% cell death after 12 hours and reached a

maximal plateau at about 68% within 20 hours post-transfection (Fig. 4.11 B). In contrast, expression of A^{213}-C-t-PAK2-myc resulted in a significant delay in the onset of programmed cell death. Indeed, at the 12 hour time point, cell death rate of cells expressing non-myristoylated C-t-PAK2 is equivalent to that of vector transfected cells (~23%) and reached ~50% only at 24 hours post-transfection. Cells co-transfected with empty vector and vector expressing EGFP presented cell death rates varying from approximately 20 to 35% between early and late time points respectively (Fig. 4.11 A and B), presumably due to the reported mild toxicity of EGFP (Goto et al., 2003). These results indicate that expression of the physiologically relevant myristoylated C-t-PAK2 induces apoptotic cell death more potently than non-myristoylated C-t-PAK2 in COS-7 cells, suggesting a key role for post-translational myristoylation in proper localization of C-t-PAK2, cell signalling and the regulation of cell death.

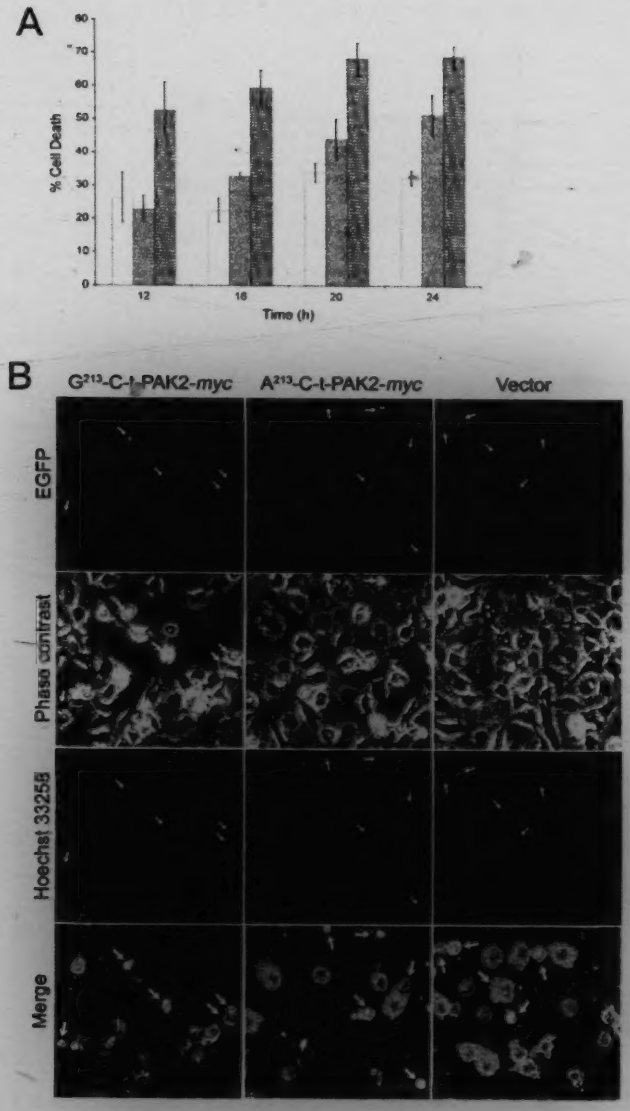


Figure 4.11. **Myristoylation enhances the capacity of C-t-PAK2 to induce cell death**. (A) COS-7 cells were co-transfected with plasmids expressing EGFP and plasmids expressing Gly^{213}-C-t-PAK2-myc (diagonally stripped bars) or A^{213}-C-t-PAK2-myc (closed bars) chimeras or vector alone (open bars). At the indicated times, cells were stained with 25 μg/ml Hoechst 33258 (Blue) for 5 min and live photomicrographs of the same field were obtained by phase-contrast and fluorescence microscopy. The percentage of co-transfected apoptotic cells for each time point was calculated as described in Materials and Methods and plotted. Data are means ± SE from three independent experiments. (B) Representative fluorescence, phase-contrast and merged images of EGFP co-transfected cells taken 24 h post-transfection. Arrows indicate apoptotic cells.

4.2.10 The apoptotic effects of C-t-PAK2 are independent of mitochondrial loss of potential and cytochrome c release

To investigate whether mitochondria are involved in the death signalling process of cells expressing the physiologically relevant form of C-t-PAK2, COS-7 cells were transiently transfected with the myristoylatable G^{213}-C-t-PAK2-myc chimers. At 16 h post-transfection, COS-7 cells expressing G^{213}-C-t-PAK2-myc undergoing programmed cell death (as judged by nuclear condensation and cellular retraction) did not release cytochrome c into the cytosol nor loose their mitochondrial potential as assessed by indirect immunofluorescence using anti-cytochrome c antibodies and the potentiometric dye MitoTracker Red CM-H_2XRos (Poot et al., 1996) (Fig. 4.12). In contrast, COS-7 cells treated with STS readily released cytochrome c (as indicated by the diffuse staining pattern) and cells treated with the uncoupling reagent para-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) readily lost their mitochondrial potential (as suggested by the absence of MitoTracker Red CM-H_2XRos staining). This suggests that the later apoptotic events stimulated by myristoylated-C-t-PAK2 (nuclear condensation, cell retraction and rounding up leading to detachment) occur downstream of the mitochondrial apoptosis commitment step corresponding to mitochondrial cytochrome c release and loss of potential. Furthermore, this also indicates that these C-t-PAK2 dependent morphological changes do not activate the intrinsic apoptotic pathway.

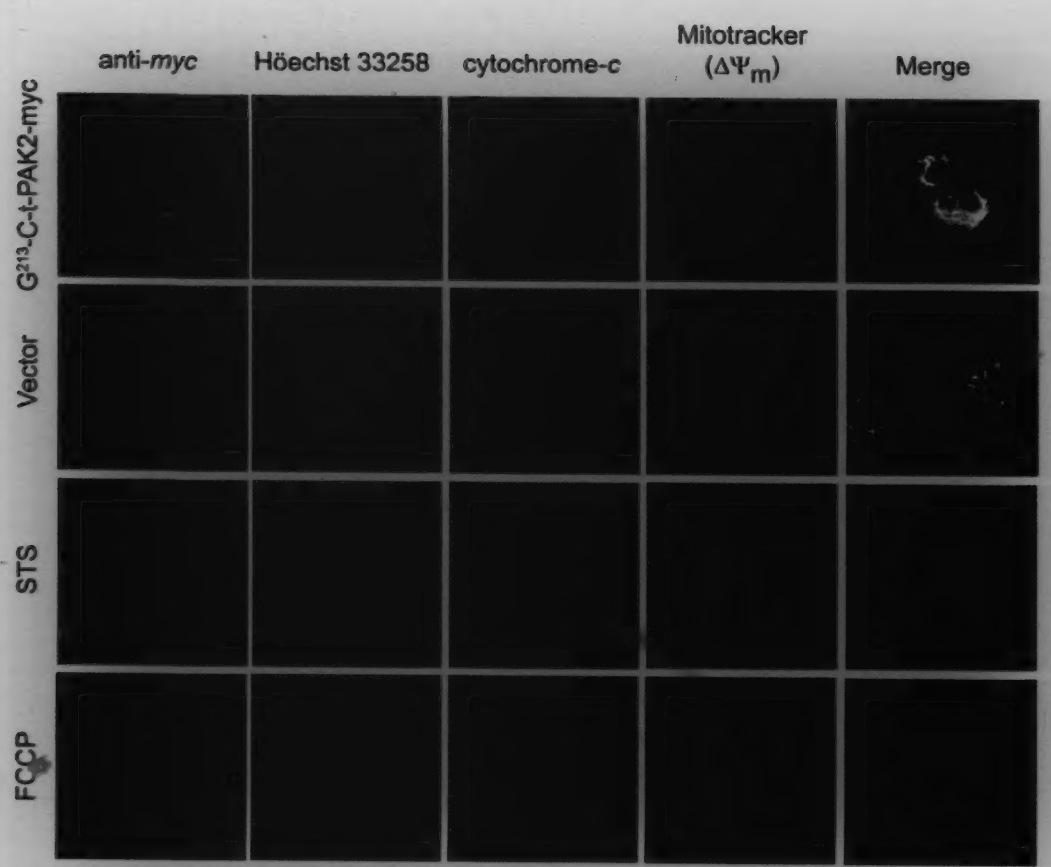


Figure 4.12. **C-t-PAK2 expression induces mitochondrial-independent cell death.** COS-7 cells were mock-transfected or transfected with either vector expressing Gly^{213}-C-t-PAK2-myc or vector alone. At 16 h post-transfection cells were either treated with 1 μM STS for 4 h, 5 μM FCCP for 15 min or left untreated. Cells were then incubated in the presence of the potentiometric dye MitoTracker Red CM-H_2XRos for 30 min. After incubation cells were processed for confocal immunofluorescence microscopy to detect C-t-PAK2-myc (green), cytochrome c (light blue), nuclei (blue) and mitochondrial membrane potential (red). The merged images are presented in the right hand column. Scale bars represent 10 μm.

4.2.11 Myristoylation of C-t-PAK2 increases JNK activation and prevents LIMK phosphorylation

In order to investigate which downstream effectors of C-t-PAK2 whose activation might induce cellular death and to determine the relative importance of C-t-PAK2 myristoylation during the activation of such pathways, the ability of myristoylatable and non-myristoylatable C-t-PAK2-myc to induce activation of c-Jun N-terminal kinase (JNK), a protein that plays crucial roles in the regulation of cell death in response to many stimuli, was compared. At 14-16 h post-transfection, COS-7 cells transfected with Gly^{213}-C-t-PAK2-myc resulted in more than a five fold increase in JNK phosphorylation when compared to vector transfected cells. In contrast, JNK phosphorylation of A^{213}-C-t-PAK2-myc transfected cells was only about three and a half fold (Fig. 4.13 A). The overall 1.5 fold increased stimulation of JNK phosphorylation by the myristoylated C-t-PAK2 over the non-myristoylated C-t-PAK2 was statistically significant ($n=4$, $p \leq 0.05$ in a Student t test). These results indicate that myristoylated C-t-PAK2 is again more effective than non-myristoylated C-t-PAK2 at inducing the stress activated signalling pathway via JNK phosphorylation, which is a key pathway regulating apoptosis in many cell types.

LIM kinases (LIMK1 and LIMK2) are serine/threonine kinases that have two zinc finger motifs, known as LIM motifs, in their amino-terminal regulatory domains (Okano et al., 1995). LIM kinases are involved in actin cytoskeletal regulation downstream of Rho-family GTPases, PAKs and ROCK (Edwards et al., 1999; Maekawa et al., 1999). Phosphorylation at the conserved Thr508 or

Thr 505 residues in the activation loop increases the LIMK activity (Edwards et al., 1999; Ohashi et al., 2000; Sumi et al., 2001). Activated LIM kinases inhibit the actin depolymerisation activity of cofilin by phosphorylation at the amino-terminal Ser 3 residue of cofilin (Arber et al., 1998; Yang et al., 1998). Cofilin plays pivotal roles in cytokinesis, endocytosis, embryonic development, stress response and tissue regeneration (Carlier et al., 1999). In response to stimuli, cofilin promotes the regeneration of actin filaments by severing pre-existing filaments (Condeelis, 2001). Cofilin binds to actin monomers and polymers and promotes the disassembly of actin filament, and phosphorylation of cofilin via LIMK inactivates this potential.

Homogenates from COS-7 cells transfected with Gly^{213}-C-t-PAK2-myc showed no increase in the phosphorylation levels of LIMKs when compared with vector-transfected cell lysates (Fig. 4.13 B). Interestingly, samples from cells transfected with the non myristoylatable A^{213}-C-t-PAK2-myc presented a significant two fold increase in LIMK phosphorylation levels, when compared to those transfected with Gly^{213}-C-t-PAK2-myc (Fig. 4.13 B). This suggests that myristoylation of C-t-PAK2 prevents LIMK phosphorylation therefore promoting cofilin-mediated cytoskeleton rearrangement through the disassembly of actin filaments.

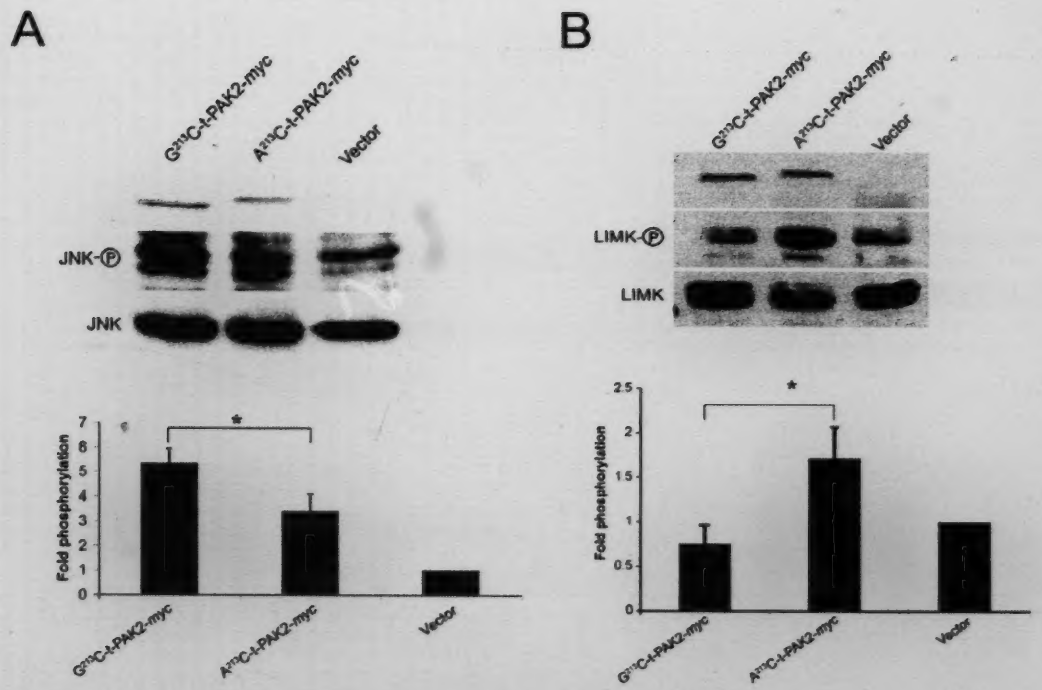


Figure 4.13. **Identification of C-t-PAK2 downstream effectors**. (A) Expression of C-t-PAK2 induces JNK phosphorylation. Cos-7 cells were transfected with either Gly^{213}-C-t-PAK2-myc, A^{213}-C-t-PAK2-myc chimeras or vector alone, 14 – 16 h post transfection cell lysates were made, separated by SDS-PAGE (12.5%) and electroblotted. Total JNK, phospho-JNK and myc were immunodetected with the proper antibodies and the intensity of the bands determined by densitometry. (B) Myristoylation of C-t-pak2 prevents LIMK (isoforms 1 and 2) activation. Cells were treated as in (A) and total LIMK, total phospho-LIMK and myc were immunodetected. Data in the histograms are presented as fold phosphorylation over vector, normalized by the corresponding total JNK or LIMK and C-t-PAK2-myc expression and represent the means ± SE of three individual experiments. *= $p \leq 0.05$.

4.2.12 Non-myristoylated A^{213}-C-t-PAK2-myc is hyperphosphorylated *in vivo*

During the experiments described above, we observed a highly reproducible slower electrophoretic mobility for the A^{213}-C-t-PAK2-myc chimera compared to Gly^{213}-C-t-PAK2-myc protein. After re-sequencing the whole construct, we discarded the possibility of a premature termination of the polypeptide and decided to investigate whether this shift in the A^{213}-C-t-PAK2-myc chimera mobility was caused by some type of post-translational modification. Interestingly, computational analyses (Blom et al., 1999) predicted 13 putative phosphorylation sites apart from the documented threonine residue 402 whose phosphorylation has been shown to be necessary for C-t-PAK2 activation (Bokoch, 2003) (Fig. 4.14 A).

Alkaline phosphatase treatment of immunoprecipitated A^{213}-C-t-PAK2-myc abolished the observed electrophoretic migration difference (Fig. 4.14 B) indicating that the non-myristoylatable form of C-t-PAK2 is hyperphosphorylated. Therefore, it appears that proper myristoylation of C-t-PAK2 might also relocalize it away from the potential detrimental or inappropriate effects of an unidentified cytosolic kinase.

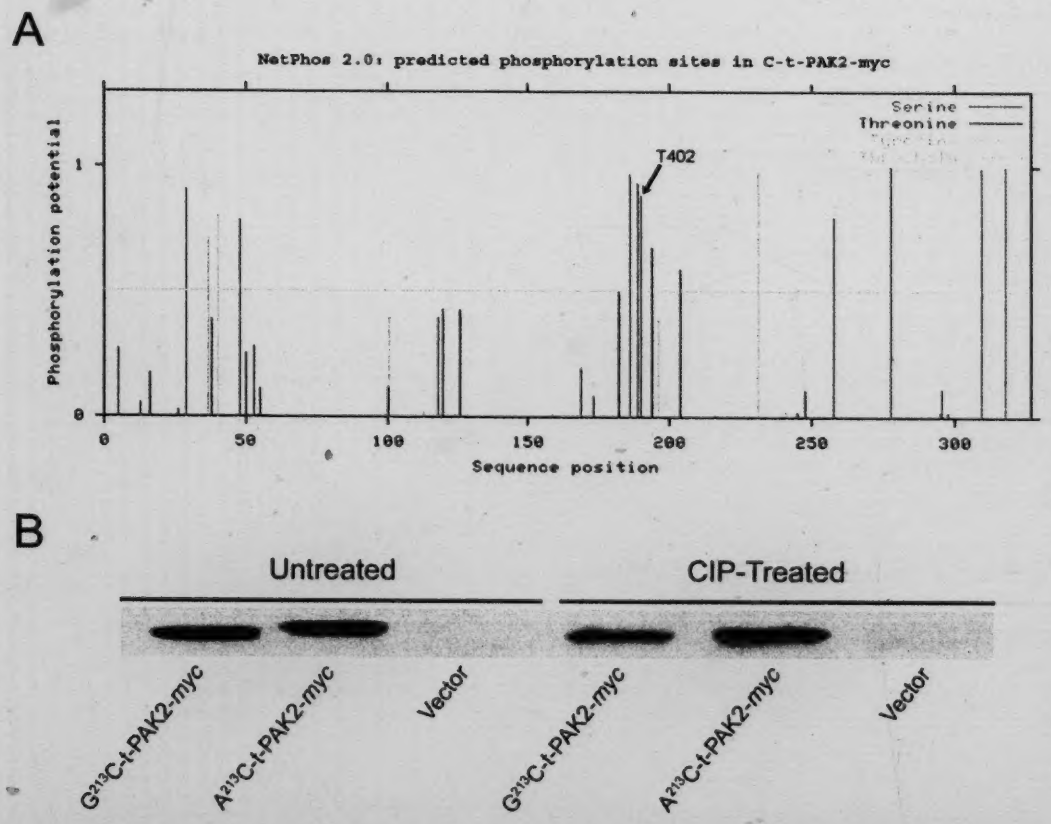


Figure 4.14. **Non-myristoylated A^{213}-C-t-PAK2-myc is hyperphosphorylated *in vivo*.** (A) Analysis of C-t-PAK2 amino acid sequence using the NetPhos 2.0 server (Blom et al., 1999) identified 14 putative serine (blue), threonine (green) and tyrosine (red) phosphorylation sites. Arrow indicates Threonine residue 402 whose phosphorylation has been shown to be necessary for C-t-PAK2 activation. (B) Cos-7 cells were transfected with either Gly^{213}-C-t-PAK2-myc, A^{213}-C-t-PAK2-myc chimeras or vector alone. Fourteen to sixteen hours post transfection the cells were lysed using CIP Extraction Buffer and 50 µg of each lysate were incubated with either calf intestine alkaline phosphatase (CIP) or water. After incubation, the samples were separated by SDS-PAGE (10 %), and PAK2 was immunodetected.

4.2.13 Phosphorylation levels do not seem to affect C-t-PAK2 kinase activity

We next sought to study whether the different phosphorylation levels found in the myristoylatable and non-myristoylatable forms of C-t-PAK2 affected the capacity of the protein to phosphorylate histone H1 *in vitro*.

To do so, COS-7 were transfected with either Gly^{213}-C-t-PAK2-myc, A^{213}-C-t-PAK2-myc or vector alone and 14 – 16 h post transfection equal amounts of total protein from each transfection lysate were incubated with anti-myc antibody in order to recover the myc chimeras. After incubation, the immunocomplexes were recovered and their ability to phosphorylate histone H1 tested.

Despite their different phosphorylation levels, neither Gly^{213}-C-t-PAK2-myc nor A^{213}-C-t-PAK2-myc differed in their kinase activity towards the substrate (Fig. 4.15) suggesting that phosphorylation levels do not affect the capacity of C-t-PAK2 to phosphorylate its substrates, at least *in vitro*.

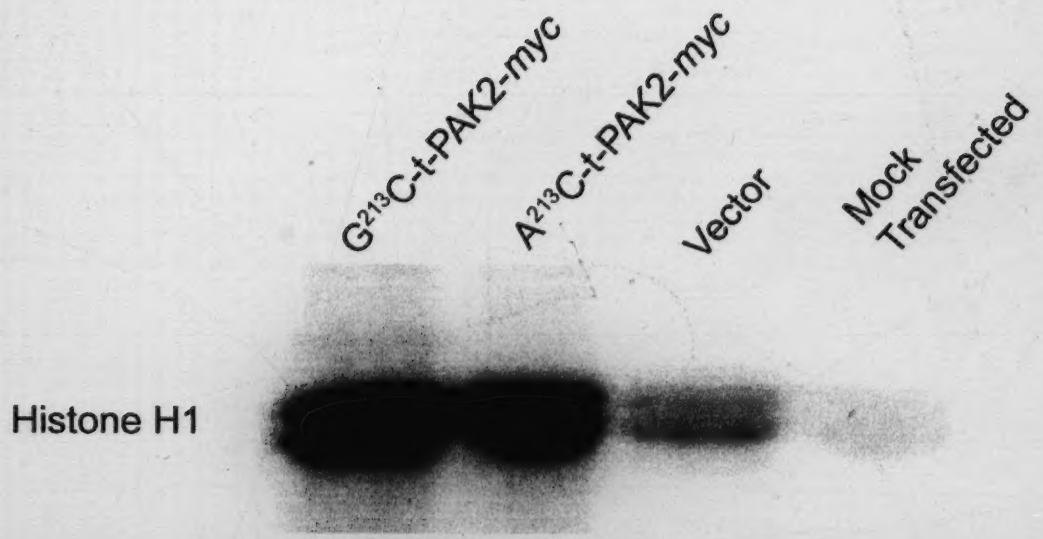


Figure 4.15. **C-t-PAK2 *in vitro* kinase activity.** COS-7 cells were mock transfected or transfected with either Gly^{213}-C-t-PAK2-myc, A^{213}-C-t-PAK2-myc chimeras or vector alone. The cells were lysed and PAK2 was immunoprecipitated. The kinase activity of the immunocomplexes was assessed by use of an in vitro kinase assay with histone H1 as the substrate as described in materials and methods. The reactions were stopped and the samples were separated by SDS-PAGE (10 %). The gels were stained, destained dried, exposed to a phosphorimager screen for 24 h and scanned.

4.3 Discussion

Ubiquitously expressed PAK2 is one of six p21 activated serine/threonine kinases involved in the regulation of various cytoskeletal functions and the only PAK cleaved by caspases in the later events of apoptosis (Bokoch, 2003). Although myristoylation is typically a co-translational process, here we demonstrate that caspase-activated C-t-PAK2 is enzymatically post-translationally myristoylated by NMT. We also show that myristoylation redirects C-t-PAK2 to membrane ruffles and endosomes where it promotes cell death without compromising mitochondrial integrity. The relocalization of myristoylated-C-t-PAK2 to membrane ruffles and endosomes is novel and contrasts with the previous demonstrations that post-translationally myristoylated t-Bid and t-actin were both found to localize to mitochondria (Utsumi et al., 2003; Zha et al., 2000).

Caspase-3 cleavage removes most of the known protein-protein interacting domains found in PAK2 (Bokoch, 2003). Interestingly, the membrane bound small GTPase Rac, a PAK2 activator, (Bokoch, 2003) was shown to be caspase-cleaved and inactivated during apoptosis (Zhang et al., 2003). Therefore, removal of the PAK2 inhibitory domain in conjunction to proper myristoylation might not only serve to activate the PAK2 kinase domain independent of small GTPases but also as a targeting signal toward regions of the cell that contain PAK2 substrates that require to be activated during apoptosis. This activation/relocation step could lead in part to a better activation of the stress activated signalling pathway as seen by the increased JNK

phosphorylation and argues in favor of a stress signalling pathway that originates at membranes.

Activated full length PAK2 has been reported to stimulate cell survival and growth (Bokoch, 2003; Jakobi et al., 2003; Misra et al., 2005; Walter et al., 1998). By redirecting the protein to specific subcellular regions, myristoylation might also prevent C-t-PAK2 from activating such survival-related pathways as the inactivation of cofilin via LIM kinases or inactivating pro-apoptotic ones such as Bad (Jakobi et al., 2003; Misra et al., 2005) and therefore generating conflicting signalling in the cells. Our findings that myristoylation of C-t-PAK2 prevented LIMK phosphorylation and that cells expressing myristoylated-C-t-PAK2 presented significant alterations in their cytoskeleton, exhibited a significant increase in the number of membrane ruffles and an apparent loss of stress fibers support this hypothesis.

Because two of the other identified caspase-cleaved post-translationally myristoylated proteins are actin itself and the actin severing protein gelsolin (Utsumi et al., 2003), our results indicate that post-translational myristoylation might play a general role in the regulation of cytoskeletal structure during apoptosis. It is well known that alteration of normal cytoskeletal dynamics, such as attenuation of actomyosin cortex contractility or disruption of the structure of microtubules, can induce apoptosis. In fact, many anti-cancer drugs (e.g. taxol, vincristine and vinblastine) exert their therapeutic actions by altering cytoskeletal dynamics of transformed cells (Pienta and Coffey, 1991; Wang et al., 1999). It is

possible that the changes in the cytoskeleton mediated by myristoylated-C-t-PAK2 kinase activity may lead to mechanical stress that triggers cell death.

Myristoylation of C-t-PAK2 might also serve to localize it away from the potential inappropriate effects of some cytosolic kinase(s), as suggested by our data showing that the electrophoretic migration of non-myristoylated A^{213}-C-t-PAK2 is altered due to hyperphosphorylation. Our phosphorylation assay suggests that the potential inappropriate effects of such putative cytosolic kinase(s) do not affect A^{213}-C-t-PAK2 kinase activity. However, since the reactions were carried out *in vitro* using an immunopurified protein and a general substrate for serine/threonine kinases that is probably not a natural substrate for this protein, we can not rule out that under physiological conditions this hyperphosphorylation is not able to alter the activity of the enzyme.

When appended to the EGFP reporter protein, the N-terminal 15 amino acid of C-t-PAK2 encompassing the myristoylation signal and a short polybasic domain is sufficient to confer a subcellular localization highly similar to that of the entire myristoylated-C-t-PAK2 protein. These new results corroborate previous results from this laboratory and those of others which suggest that various short N-terminal domains encoding for combinations of myristoylation and polybasic signals or myristoylation and palmitoylation signals are endosomal and plasma membrane targeting signals (McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001; Resh, 2004).

Membrane localization of C-t-PAK2 and its ability to induce cell death as judged by the nuclear condensation/fragmentation and retraction/roundness of

live transfected cells are significantly reduced when myristoylation is abolished. This demonstrates once again the importance of myristoylation in proper localization and function of various proteins (Resh, 2004) as well its novel involvement in the regulation of apoptosis.

Our findings also agree with the results obtained by Bisson *et al.* who showed that expression of an N-terminally HA-tagged caspase-activated C-terminal Xenopus PAK1 (HA-C-t-xPAK1) in cells did not induce apoptosis unless a –CAAX prenylation/membrane tethering signal was appended to it (Bisson et al., 2003). Under the light of our new findings we can now explain why this artificial membrane tethering was required. *Xenopus* PAK1 (xPAK1), the functional equivalent to human PAK2 (hPAK2) (Bisson et al., 2003) also contains a putative myristoylation signal downstream of its caspase-cleavage site (SATD-**G**AES**S**VDKTKKKPK) followed by a short polybasic domain in which, interestingly, the positions of lysine residues downstream of the myristoylation signal are conserved compared to those found in hPAK2. In their elegant study, Bisson *et al.* abrogated the internal post-translational myristoylation by appending an N-terminal HA-tag to C-t-xPAK1, thus preventing post-translational myristoylation and abolishing its capacity to associate with membranes (Bisson et al., 2003).

Importantly, with the exception of the work by Rudel *et al.* where cell death upon expression of C-t-PAK2 was assayed but the protein's sub-cellular localization was not assessed (Rudel et al., 1998), all the experiments in published articles were performed with non-physiological forms of C-t-PAK2

bearing a variety of *N*-terminal epitope tags, therefore abolishing post-translational myristoylation (Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997; Rudel and Bokoch, 1997). Although their overall findings corroborate ours on the importance of C-t-xPAK1/C-t-PAK2 in apoptosis, theirs might be due to overexpression of the non-physiological non-myristoylated form of C-t-xPAK1/C-t-PAK2.

The cleavage of PAK2 by caspase-3 is a relatively late event in the onset of apoptosis and occurs once the cell has passed the critical apoptotic commitment point, which involves mitochondrial cytochrome c release and ensuing activation of executioner caspase-3. Our exogenous expression of C-t-PAK2 (no more than two fold) in COS-7 cells induced striking cell retraction and nuclear condensation leading to cell death without disrupting mitochondrial potential or inducing cytochrome c release. These results pinpoint the action of myr-C-t-PAK2 downstream of the mitochondrial commitment step and the formation of the apoptosome. Our data is also supported by the work of Lee *et al.*, Jakobi *et al.* and Koeppel *et al.* who showed that expression of C-t-PAK2 lead to adherent cell shrinking, rounding up and nuclear condensation without exhibiting classical hallmarks of apoptosis (no phosphatidylserine externalization and absence of TUNEL reactivity) (Jakobi et al., 2003; Koeppel et al., 2004; Lee et al., 1997).

Our results therefore suggest that deregulation of PAK2 kinase activity leading to altered phosphorylation of protein(s) involved in the regulation of cytoskeleton structure is sufficient to induce cell death. It is well known that

alteration of normal cytoskeletal dynamics, such as attenuation of actomyosin cortex contractility or disruption of the structure of microtubules, can induce apoptosis. In fact, many anti-cancer drugs (e.g. taxol, vincristine and vinblastine) exert their therapeutic actions by altering cytoskeletal dynamics of transformed cells. It is possible that the changes in the cytoskeleton mediated by C-t-PAK2 kinase activity may lead to mechanical stress that triggers a signalling cascade that induces nuclear condensation or directly acts on the nuclear envelope causing the chromatin to collapse. In either case, the final result would be a potential transcriptional shut down that would eventually lead to cell death.

Mechanistically, the task of linking myr-C-t-PAK2 kinase activity to potential substrates is a difficult one. PAK2 has only a loose optimal "KRES"-phosphorylation motif suggesting the potential importance of local 3D structure, protein-protein interactions and intracellular localization of the substrate in its phosphorylation (Bokoch, 2003). In addition, the substrate specificity and specific activity of myristoylated-C-t-PAK2 is potentially different than that of full length PAK2 once the regulatory domain has been proteolytically removed and the PAK2 kinase domain is relocalized to membranes. So far, Bisson *et al.* (Bisson et al., 2003) provided the only study confirming the identification of a substrate for a caspase-activated PAK (C-t-xPAK1) as the regulatory myosin light chain (R-MLC) which was shown to be phosphorylated on Thr-18 and Ser-19. R-MLC is known to increase contractility (Bokoch, 2003) and was suggested to be involved in membrane blebbing (Mills et al., 1998). In COS-7 cells expressing myristoylated-C-t-PAK2, but not in cells expressing non-

myristoylatable C-t-PAK2, we detected significant actin structure reorganization based on fluorescent-phalloidin staining data. The data showing disruption of actin stress fibers into cytosolic clusters and increased actin presence in membrane ruffles in these cells further strengthen the argument in favor of a critical role for post-translational myristoylation of C-t-PAK2 in proper regulation of actin structure during programmed cell death.

In the present study, we also show that there are at least nine post-translationally myristoylated proteins in Jurkat cells undergoing apoptosis. These results indicate that there are several cases of post-translational myristoylation of proteins during the onset of apoptosis and that this phenomenon might play an important novel role in the regulation of apoptosis.

Our results indicate that post-translational myristoylation of C-t-PAK2 might play a role in the regulation of cytoskeletal structure during apoptosis and that this perhaps represents one of the main regulatory roles for post-translationally myristoylated proteins. This possibility is further strengthened by the fact that two of the other identified caspase-cleaved post-translationally myristoylated proteins are actin itself and the actin severing protein gelsolin.

The reason for the relocalization of the constitutively active PAK2 kinase activity in the form of myristoylated-C-t-PAK2 from the cytosol to endosomes or membrane ruffles still remains to be elucidated. Whether myristoylated-C-t-PAK2 potentiates death signals, inhibits survival signals at these locations or simply alters local cytoskeleton structure in order to favor apoptotic body removal requires further investigation.

Since myristoylated-C-t-PAK2 dependent events mediate cell death downstream of the mitochondrial commitment step and many cancer cells (e.g. B cell lymphomas) over-express anti-apoptotic Bcl-2 family members which prevent the triggering of apoptosis at the mitochondrial level, finding novel ways to reactivate PAK2 in cancer cells or deliver constitutively active myr-C-t-PAK2 into cancer cells might lead to potential new anti-cancer therapies.

CHAPTER 5

DISCUSSION

5.1 Overview

Although the knowledge on protein fatty acylation has been growing for more than 20 years, it is only recently that the functional significance of this modification has started to be fully appreciated. It is now clear that myristoylation and palmitoylation influence a wide spectrum of structural and functional features of proteins. Fatty acylation of proteins appears to play critical roles in several protein kinases or their substrates, cell surface receptors, and guanyl nucleotide binding proteins. Several oncogenic proteins have been found to undergo fatty acylation and to require this modification to exert their biological effects (e.g. Src and Ras). Therefore, fatty acylation appears to be an important regulatory step controlling the tethering of these proteins to various membranes or membrane-associated effector elements. In addition, fatty acylation plays an essential role in the sorting of proteins between membrane organelles and within microdomains of individual membrane compartments, as well as in the modulation of certain protein-protein interactions (Berthiaume, 2002; Corvi et al., 2001; Greeve, 2005; Magee and Seabra, 2005; Mann and Beachy, 2004; McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001; Miura et al., 2006; Resh, 2004; Sakurai and Utsumi, 2006; Vilas et al., 2006; Yanai et al., 2006). The mechanisms underlying the fatty acylation-dependent regulation of cellular signalling pathways and trafficking of acylated proteins remain unclear and therefore further insights are needed for a better understanding of them. In light of the potential importance of protein fatty acylation in the establishment of

transmembrane signalling pathways it is also important to identify new fatty acylated proteins involved in the regulation of cellular processes.

This thesis work focused on studying two important and poorly characterized regulatory aspects of the protein fatty acylation process: 1) The role of palmitoylation in subcellular localization, sorting and assembly of apolipoprotein B, an essential component of LDL, VLDL and chylomicrons and 2) The effects of the recently described process of post-translational myristoylation on the localization and activity of caspase-3 cleaved PAK2, a protein that plays an important role in the reorganization of the cytoskeleton.

5.2 ApoB, a case study for the role of fatty acylation as a positive signal responsible for the intracellular sorting and localization of lipidated secreted proteins

Previous work carried out in our laboratory showed that non-palmitoylated apoB-29 lost its proper distribution along the secretory pathway and failed to properly assemble cholesterol and triglyceride displaying an 80% reduction of these lipids in lipoprotein particles (Zhao et al., 2000). The work presented in this thesis has increased the knowledge on the role for palmitoylation in quality control, assembly and secretion of apoB-containing lipoprotein particles by showing that palmitoylation of apoB occurs early in its biogenesis, stimulates ER-Golgi transport rate of apoB-29, doubles apoB-29 secretion efficiency and appears to be involved as a quality control step favoring dissociation of apoB-29 from the ER chaperone CLX. In addition, non-palmitoylated apoB-29 co-

localized extensively with constitutively secreted protein transferrin while palmitoylated apoB-29 showed only partial co-localization and appeared to be enriched in extensions of the ER.

Physiologically speaking, it has been long established that when availability of neutral lipids is poor most of apoB is degraded (Davidson and Shelness, 2000; Davis, 1999; Ginsberg, 1997) and when levels of neutral lipids are increased, the apoB secretion efficiency also increases (Davis, 1996; Fisher et al., 1997; Gordon et al., 1994). Since palmitoyl-CoA is the substrate of key neutral lipid synthetic enzymes such as diacylglycerol acyltransferase and acyl-CoA cholesterol acyltransferase as well as for the palmitoyl acyltransferase (PAT) that palmitoylates apoB, an emerging concerted model could be implied in which increased levels of palmitoyl-CoA could stimulate the activity of neutral lipid synthetic enzymes as well as that of the apoB PAT thereby increasing the apoB rate of ER-Golgi transport, reducing apoB degradation and overall increasing secretion efficiency.

Since the increase in the retention time of non-palmitoylated apoB-29 was not due to a severe difference in folding, palmitoylation appears to be involved in the fine tuning or quality control of the assembly and folding of the nascent apoB–containing lipoprotein particle prior to its secretion. In addition to apparently promoting the dissociation of WT apoB-29 from CLX, palmitoylation of apoB-29 might also act as an ER exit signal as it stimulates ER to Golgi transport and favors concentration of palmitoylated apoB-29 to extensions of the ER (Zhao et al., 2000). Thus, the fact that secreted lipoprotein particles

containing non-palmitoylated apoB-29 have a smaller lipid core and that the localization of these lipoprotein particles does not coincide with the localization of the palmitoylated apoB-29, suggest that palmitoylation may act as an intraorganelle sorting signal directing the protein towards specific regions of the secretory pathway where the lipid loading machinery resides. This thesis work demonstrates, for the first time, the involvement of protein palmitoylation as an active signal of the ER protein sorting and folding process undergone by a secreted protein, in this case, apoB.

Interestingly, the non-palmitoylated apoB-29 containing lipoprotein particles appear to be sorted without the concentration step in the ER extension and secreted in a more constitutive fashion, like transferrin. The timing of palmitoylation of apoB-29 agrees with its key role in the folding and processing of the protein, since it occurs early in the biosynthesis but it is also present further in the secretory pathway. Several other proteins were shown to be palmitoylated at specific stages in their maturation process. In confluent chicken embryo fibroblasts infected with influenza virus, palmitoylation of hemagglutinin (HA) was found to occur in the *cis*-Golgi network after the trimerization of the protein is completed in the ER (Veit and Schmidt, 1993). Furthermore, Schmidt and Schlesinger showed that palmitoylation of vesicular stomatitis and Sindbis Virus membrane glycoproteins occurs at a specific time in the post-translational maturation process. For the vesicular stomatitis virus, glycoprotein acylation occurred in the ER, whereas the Sindbis virus was acylated in the Golgi apparatus (Schmidt and Schlesinger, 1980).

The presence of this acylation activity in the ER and in the Golgi compartments may explain the steady state distribution of both palmitoylated and non-palmitoylated forms of apoB-29. Indeed, from our immunofluorescence data it appears that once palmitoylated in the ER, apoB-29 seems to be actively concentrated in ER subcompartments, efficiently transported to the Golgi and rapidly cleared from this location en route to the cell surface. The fact that non-palmitoylated apoB-29 appears to be concentrated in the Golgi is due to the lesser relative amount of Golgi membranes present in the cells (Warren and Mellman, 1999). Several proteins going through the secretory pathway have been shown to be acylated. These include mostly viral proteins and cell surface receptors. Examples of viral acylated proteins include Sindbis virus E2 glycoprotein (Ivanova and Schlesinger, 1993), haemagglutinin (HA) of type A influenza viruses (Portincasa et al., 1992) and the cytoplasmic domain of the HIV-1 envelope glycoprotein (gp160) (Rousso et al., 2000). Examples of acylated cellular receptors include the mannose 6-phosphate receptor (Schweizer et al., 1996), the human transferrin receptor (Alvarez et al., 1990) and many G protein coupled receptors (GPCR) such as the vasopressin V1a receptor (Hawtin et al., 2001), the muscarinic acetylcholine receptor m2 subtype (m2 receptor) (Hayashi and Haga, 1997) and the human endothelin receptor A (ETA) (Horstmeyer et al., 1996). In several cases, it has been demonstrated that acylation of these proteins is essential for their proper trafficking and sorting. Indeed, the Lutropin/Choriogonadotrophin receptor (LH/CGr) is a member of the GPCR family and contains two C-terminal cysteine residues that are

palmitoylated. Zhu and co-workers demonstrated that the non-palmitoylated form of the LH/CGr, although capable of binding human CG and transducing the cAMP signal, is trapped intracellulary and is barely detected in the surface of cells (Zhu et al., 1995). The trafficking of another GPCR family member, CCR5, which binds several CC chemokines and is also the principal co-receptor for macrophage-tropic strains of HIV (Berger et al., 1999; Littman, 1998) has been shown to be affected by the lack of palmitoylation. Indeed, Blanpain *et al* found that disruption of CCR5 palmitoylation resulted in a strong reduction of its surface expression in CHO-K1 cells via altered intracellular trafficking and that HIV entry into cells expressing the mutated non-palmitoylated CCR5 receptor was reduced by 50% (Blanpain et al., 2001). They also found that mutant, non-palmitoylated CCR5, was synthesized in CHO-K1 cells but was sequestered largely in the ER or Golgi areas and that the diffusion of this mutated receptor along the ER was severely affected. Interestingly, many of the functions of this receptor, such as chemokine binding and activation of G_i-mediated signalling pathways were not affected when palmitoylation was abolished (Blanpain et al., 2001). Likewise, Tanaka *et al.* have shown that abolition of palmitoylation of the human thyrotrophin receptor (TSHr) by site directed mutagenesis does not affect the binding affinity of the receptor for TSH neither the cAMP response to the ligand nor the homologous desensitization and TSH-induced internalization of the receptor (Tanaka et al., 1998). However, maturation of the non-palmitoylated receptor from its ~95 kDa precursor to the ~100 kDa mature form was significantly slower, thus delaying the cell surface expression of non-

palmitoylated TSHr (Tanaka et al., 1998). The fact that ligand binding and signalling function of TH/CG, CCR5 and TSH GPCR are not affected by loss of palmitoylation suggest that these membrane proteins are properly folded. Therefore, it appears that palmitoylation might represent a positive transport signal to the plasma membrane in those GPCR. In addition, palmitoylation might also act as a transport signal in different types of receptor as well. In the case of the cation-dependent mannose 6-phosphate receptor, mutation of the palmitoylated cysteine residue 34 to alanine in the receptor's cytoplasmic tail resulted in an abnormal receptor trafficking resulting in the gradual accumulation of the mutant receptor in dense lysosomes as well as the loss of ability to sort newly synthesized cathepsin D to those organelles (Schweizer et al., 1996).

Altogether and in addition to our findings on apoB palmitoylation, these observations support an important and perhaps ubiquitous role for palmitoylation as a positive signal responsible for the intracellular sorting and localization of several receptors and secreted proteins.

Furthermore, this new role might not be necessarily restricted to receptors and secreted proteins since palmitoylation of some signalling proteins found at the surface of the components of the secretory pathway was shown to be essential for localizing these proteins to the inner leaflet of the plasma membrane. Indeed, non-palmitoylated H-Ras proteins were shown to accumulate mostly on ER membranes and failed to reach the plasma membrane (Apolloni et al., 2000; Chiu et al., 2002; Choy et al., 1999). These

results provide yet another example in which palmitoylation could act as a novel type of transport signal.

Based on our results and on the evidence above, we can envision some possible mechanisms for this new fatty acylation-dependent regulation of protein transport along the secretory pathway. In these processes, palmitoylation may facilitate the sorting of the newly folded proteins by favoring their dissociation from the ER folding/sensing machinery, or, in concert with its effect on the folding, it could also preferentially bring the folded nascent proteins to ER exit sites. Either processes would contribute to reduce the ER residency time of the given acylated protein, therefore decreasing its possible degradation and increasing its secretion efficiency (Fig. 5.1). The means by which palmitate may mediate this "active sorting" to ER extensions still remains unclear, but basically four situations can be hypothesized. 1) Blocking of the free thiol via palmitoylation could favor the dissociation of the nascent protein from quality control chaperones such as PDI once the polypeptide has reached a proper conformation. 2) Palmitoylation through its added hydrophobicity could favor partitioning into a specialized lipid environment of the ER. 3) The protein-palmitoyl moiety could be recognized by a yet unknown receptor, which would reversibly shuttle between the main ER and ER subcompartments enriched in neutral lipid loading machinery. 4) The fourth situation is a variation of the last two, in this case the lipid modification induces a slight conformational change in the protein that exposes a ligand for a membrane receptor which then binds to the protein and relocalizes it to its final destination.

In such models, the regulation of a putative palmitoyl acyl transferase activity would then be critical for proper control of the acylated protein movement along the secretory pathway. In the specific case of apoB-29, palmitoylation could also be directing the protein towards specific PDI enriched regions of the ER as seen in Zhao *et al* (Zhao et al., 2000). Interestingly, PDI is a subunit of MTP, which has been proven to be involved in the neutral lipid loading of the apoB precursor. Thus, palmitoylation of apoB-29 could bring the nascent protein to the "lipid loading" machinery.

Further studies and characterization of this novel mechanism as it relates to apoB intracellular transport may lead to the development of new therapeutic drugs capable of inhibiting or decreasing apoB's palmitoylation by blocking the activity of the palmitoyl acyl transferase or enhancing the thioesterase activity of the enzyme responsible for its depalmitoylation. Once palmitoylation levels are decreased, this would likely increase ER retention of apoB-containing lipoproteins therefore increasing degradation rate and reducing the circulating levels of apoB.

In conclusion, this work has identified and characterized a novel and perhaps ubiquitous fatty acylation-dependent sorting mechanism that is likely involved in the sorting of a wide variety of proteins, including receptors, signalling and secretory proteins.

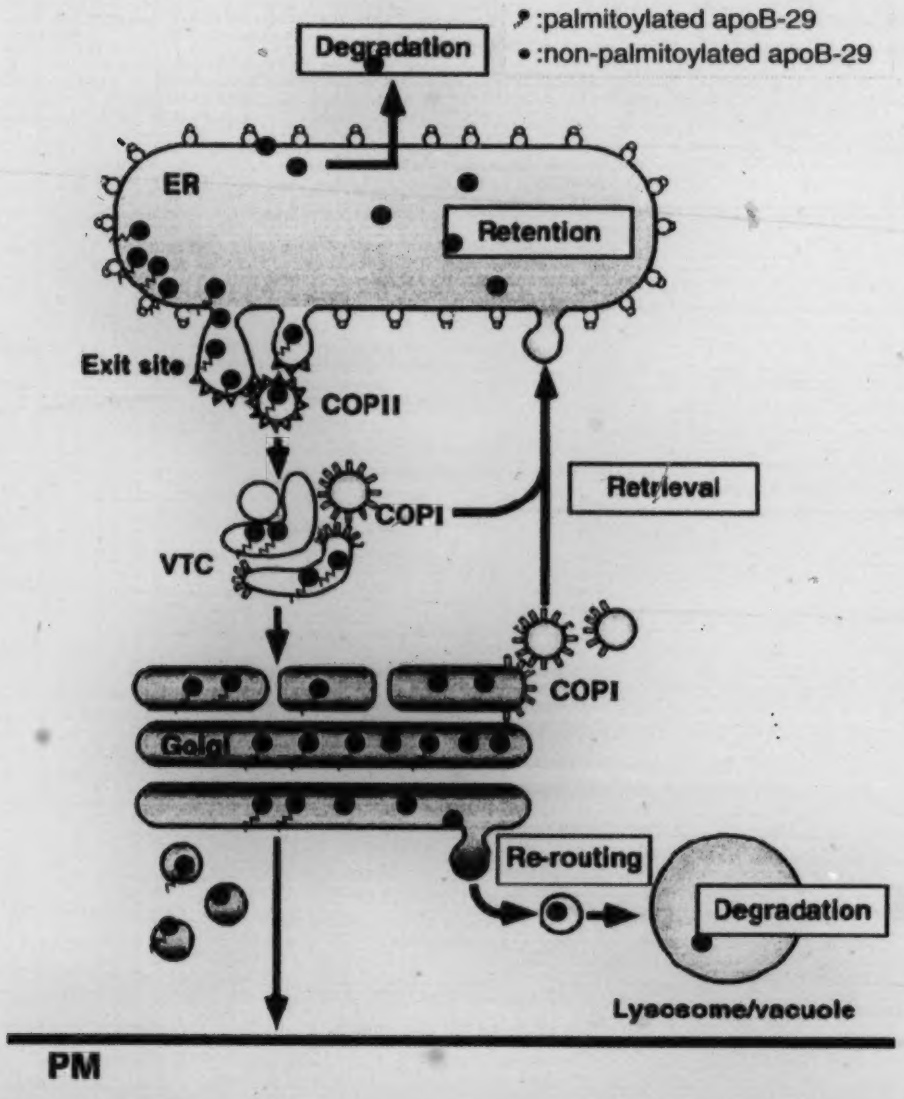


Figure 5.1. **Proposed model for the palmitoylation-dependent sorting of apo-29.** Palmitoylation of cysteine residue 1085 may facilitate the sorting of the newly folded protein by favoring dissociation from the ER folding/sensing machinery, or, in concert with its effect on folding, it could also preferentially bring the folded nascent protein to ER exit sites.

5.3 Post-translational myristoylation of PAK and the modulation of the cellular death process by fatty acylation

The regulated proliferation of metazoan cells requires a balance of mitogenic signals and survival signals. These survival signals are typically supplied by specific growth factors and cell-matrix contacts that prevent programmed cell death.

The mitochondrion is at the center of the apoptotic process since it contains key cell death effector proteins such as AIF and cytochrome c that are released upon stimuli from the extrinsic or intrinsic pathways. The release of such mitochondrial proteins is promoted by pro-apoptotic members of the Bcl-2 family such as Bax, and caspase-8 cleaved Bid (tBid). Until recently, the mechanism of tBid relocation to mitochondria was a mystery. In 2000, Zha *et al* demonstrated that upon induction of apoptosis, Bid was cleaved by caspase-8 into 7 and 15 kDa fragments resulting in the exposure of a myristoylatable glycine residue at the N-terminus of the C-terminal 15 kDa fragment (tBid), which was myristoylated by N-myristoyl transferase (NMT) in a novel post-translational fashion (Zha et al., 2000). With the added hydrophobicity of the myristoyl moiety, myristoylated tBid is targeted to the mitochondria where it potentiates cytochrome c release 3-4 fold over non-myristoylated-tBid. The discovery of post-translational myristoylation of caspase-cleaved Bid was an exciting finding since, until that moment, myristoylation was considered to be exclusively a co-translational process. This new finding also suggested an important role for protein fatty acylation in the regulation of the apoptotic process.

Computational analyses performed by our laboratory identified a putative myristoylation site adjacent to the caspase-3 cleavage site in PAK2. The findings presented in this study show that: 1) C-t-PAK2 incorporates [^{3}H]myristate at glycine residue 213 via an amide bond in a post-translational fashion, in contrast to the typical co-translational myristoylation process, 2) Myristoylation and the adjacent polybasic domain of C-t-PAK2 are sufficient to redirect EGFP from the cytosol to membrane ruffles and internal membranes, 3) Membrane localization and the ability of C-t-PAK2 to induce cell death are significantly reduced when myristoylation is abolished, 4) Proper myristoylation-dependent membrane ruffles and endosomes localization of C-t-PAK2 significantly increased signalling through the stress-activated JNK signalling pathway, which often regulates apoptosis and favored the cofilin-dependent cytoskeletal rearrangement process by preventing C-t-PAK2-dependent LIMK activation, 5) Electrophoretic migration of non-myr-C-t-PAK2 is altered and is due to hyperphosphorylation of the non-physiological non-myristoylated form of C-t-PAK2 suggesting that myristoylation might relocate C-t-PAK2 away from this "inappropriate" cytosolic kinase activity, and 6) C-t-PAK2 promotes cell death without compromising mitochondrial integrity, suggesting a role for C-t-PAK2 as an "executioner kinase" acting downstream of the mitochondrial commitment point.

Taken together, these results and the work done by other research groups suggest that post-translational myristoylation of caspase-cleaved proteins (e.g. PAK2, actin, gelsolin) contribute to the regulation of apoptosis. To further

illustrate this possibility, this work also demonstrates the existence of at least 9 post-translationally myristoylated proteins in Jurkat cells undergoing apoptosis.

Based on the information presented, a model where post-translational myristoylation acts as a regulator of caspase-cleaved protein localization and function can be envisaged (Fig. 5.2). In this model, post-translational myristoylation of specific caspase-cleaved proteins during apoptosis is part of a two-signal system that allows interaction with specific membranes and membrane subdomains via specific lipid interactions or interaction with resident membrane proteins. By restricting localization of the post-translationally myristoylated protein to certain membranes/membrane domains, myristoylation can 1) Direct the modified proteins to specific regions of the cell where they can interact with new partners and trigger signalling events that are only required under apoptotic conditions, 2) Localize them away from the potential inappropriate effects of cytosolic proteins, 3) Prevent them from interacting with substrates whose activation is not required for the onset of the apoptotic process, or 4) Increase the local specific activity of certain proteins and potentiate their effects. In the absence of myristoylation, these proteins are less efficient and the apoptotic process is slower.

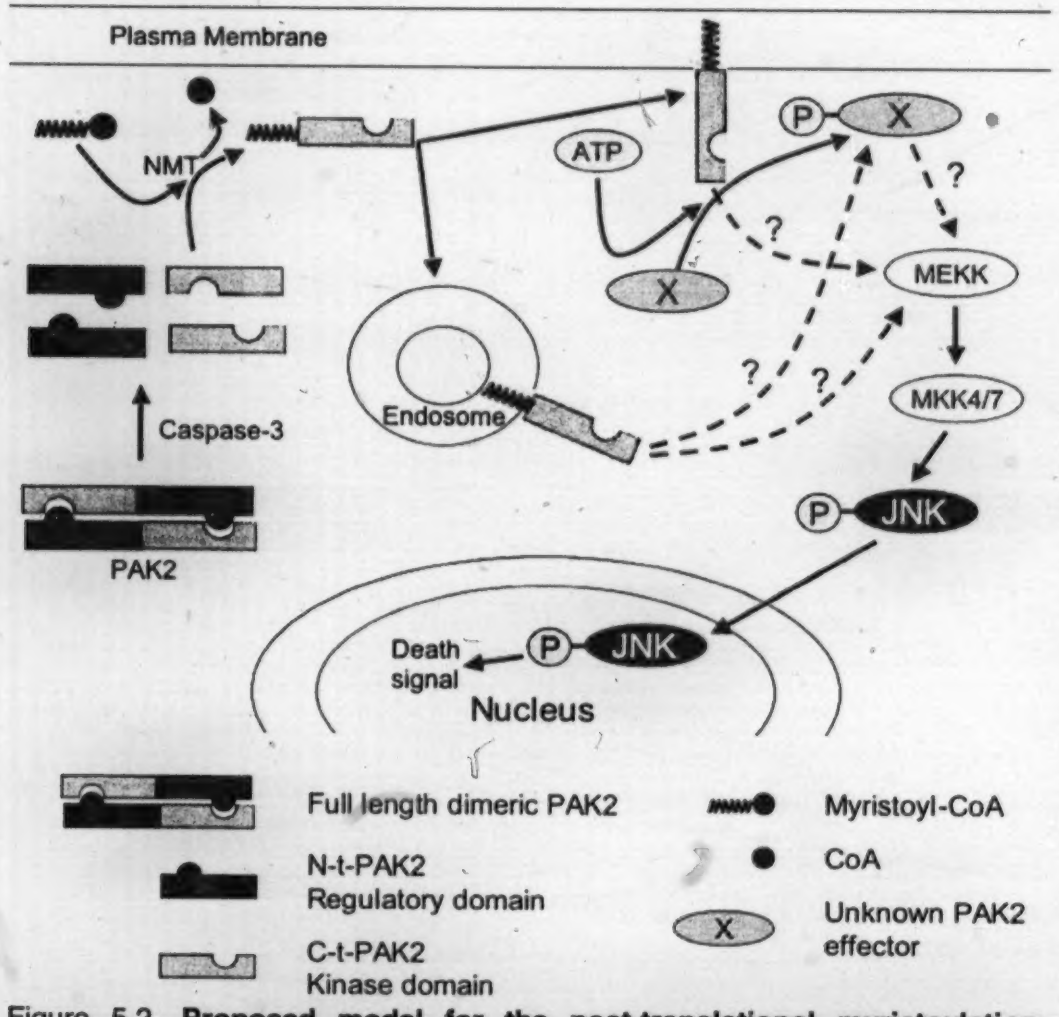


Figure 5.2. **Proposed model for the post-translational myristoylation-dependent regulation and function of caspase-activated PAK2 during apoptosis.** Post-translational myristoylation directs C-t-PAK2 to certain membranes/membrane domains where this protein may either interact with new partners and trigger signalling events that are only required under apoptotic conditions or increase the local specific activity of certain proteins and potentiate their effects.

Overall, the work presented within this section of the thesis indicates that post-translational myristoylation likely represents a novel and general mode of cell death regulation.

5.4 Future directions

This work has demonstrated the importance of fatty acylation in the regulation of apoptosis and in the secretion of proteins. Although the experiments presented here have provided the basis for this assertion, they have also raised many questions that are important for an in depth understanding of the role of protein fatty acylation in the control of such metabolic processes.

5.4.1 Assembly and secretion of apoB-containing lipoprotein particles

In order to further elucidate the mechanism of palmitoylation-dependent localization, sorting and assembly of apoB it is important to investigate the role of chaperones, folding sensors, and intracellular levels of palmitoyl-CoA in the biogenesis and secretion of the protein.

The work performed by Zhao *et al* in our laboratory (Zhao et al., 2000) and Vukmirica et al (Vukmirica et al., 2003) indicated the presence of multiple palmitoylation sites different from cysteine residue 1085 within apoB. Interestingly, the amino acids surrounding the first three free cysteine residues of human apoB are very similar (LSC^{1085}D, LSC^{1395}D and LSC^{1478}Q). The amino acids surrounding the fourth free cysteine residue (LKC^{1635}S) are

somewhat less related while the sequences of the remaining five free cysteines present in human apoB were not similar to the first four.

It is possible that elements of the cellular quality control machinery might recognize this putative "LSCD" palmitoylation motif of apoB and relocalize the protein to subcompartments of the ER enriched in PDI and calreticulin that are the proposed sites for addition of neutral lipids to nascent apoB. To test whether an apoB palmitoylated sequence acts as a targeting/localization sequence within the ER, palmitoylated and non-palmitoylated apoB sequences of various lengths could be appended to a reporter protein such as transferrin and their colocalization with different secretory pathway markers as well as endogenous secreted proteins studied.

Finally, it is also very important to identify the PAT responsible for apoB palmitoylation since this might lead to the development of specific inhibitors that could result in lower blood cholesterol and triglycerides and, therefore, help to reduce the chances of developing metabolic syndrome. The metabolic syndrome, which is very common in the general population, is defined by the concomitant occurrence, of several classic cardiovascular risk factors, such as type 2 diabetes, hypertension, high triglycerides and low HDL. Central obesity and insulin resistance, which are the two underlying disorders of the syndrome, are further risk factors for cardiovascular disease (Bonora, 2006).

Developing compounds capable of inhibiting the PAT responsible for the palmitoylation of apoB could benefit our over-nourished society where obesity

and the associated development of type II diabetes are acquiring epidemic proportions (Zimmet et al., 1999).

5.4.2 Post-translationally myristoylated C-t-PAK2 and programmed cell death

In addition to establishing the contribution of the newly discovered post-translationally myristoylated proteins to biochemical and morphological changes occurring during apoptosis, future work will be focused in elucidating the molecular mechanisms by which C-t-PAK2 regulates programmed cell death. As such, the data presented in this work have raised several questions worth pursuing.

1) This work has shown that expression of myristoylated-C-t-PAK2 in COS-7 cells leads to apoptotic morphology, nuclear condensation, cell detachment and cell death. On the other hand, non-myristoylated C-t-PAK2 was not as potent at inducing cell death. These results suggest that re-localization of constitutively active C-t-PAK2 from the cytosol to membranes is sufficient to induce cell death. Consequently, it is important to investigate why C-t-PAK2 requires a change in subcellular localization for optimal function and identify differentially phosphorylated proteins.

2) During apoptosis, a significant number of proteins are cleaved by caspases and, as shown in this work, several of them are post-translationally myristoylated. This suggests that PAK2 is probably only one of many proteins whose acylation-dependent relocation is used to regulate the execution of the cell death program. To further illustrate the importance of PAK2, PAK2 $^{-/-}$ mice

die during early embryonic stage suggesting that this protein is very important for proper animal development (Bokoch, 2003). Therefore, it would be very interesting to address the regulation of apoptosis in mouse embryo fibroblasts devoid of PAK2 and to address how critical is the action of myristoylated C-t-PAK2 during this process by exogenously expressing various C-t-PAK2 constructs in these cells.

3) Because deregulated cell proliferation and inhibition of apoptosis lie at the heart of tumor development, the signalling pathways involved represent obvious targets for therapeutic interventions. Since this study has shown that the sole expression of C-t-PAK2 in COS-[illegible] cells leads to cell death, it will be important to examine whether the exp[illegible] of this protein is also able to reactivate the apoptotic program of canc[illegible] using a gene therapy approach.

4) In humans, there are two isoforms of [illegible]MT1 and hNMT2 (Giang and Cravatt, 1998). Although this work has proven that C-t-PAK2 is myristolylated by NMT, the identity of the isoform still remains to be elucidated. Consequently, it is important to identify which NMT isoform is responsible for the myristoylation of C-t-PAK2 in order to further understand the role of this modification during the apoptotic process.

5) In normal, healthy cells, PAK2 exists as a cytosolic homodimer, with the amino-terminal regulatory domain of one molecule bound and inhibiting the carboxy-terminal kinase domain of the other. During apoptosis PAK2 is cleaved by caspase-3 (Bokoch, 2003; Jakobi et al., 2003; Rudel and Bokoch, 1997). This cleavage removes the 28kDa N-terminal regulatory domain and generates

a constitutively active 34 kDa C-terminal fragment (C-t-PAK2) (Rudel and Bokoch, 1997). Interestingly, the fate of the N-terminal regulatory domain is unknown. Thus, studies should be performed in order to elucidate if the N-terminal regulatory domain remains bound to C-t-PAK2 after cleavage, like BID, and to establish whether this regulatory fragment plays any role during the apoptotic process.

5.5 Conclusions

This study on the role of protein fatty acylation in the control of cellular metabolic processes has identified palmitoylation and post-translational myristoylation as versatile modifications that can alter protein localization, secretion and function. The results presented here show that fatty acyl modification of proteins play critical roles in membrane association and targeting and lead to a better understanding of the regulation of cellular signalling pathways and the intracellular localization of proteins.

The findings within this work will contribute to the better mechanistic understanding of apoptosis regulation in cancer and will provide new insights on spatial and temporal palmitoylation-dependent regulation of protein transport along the secretory pathway. Such understanding of cell death mechanisms and secretion of apoB-containing lipoprotein particles are important for the development of new cures aimed at reactivating apoptotic pathways in cancer cells and to the design of novel cholesterol and triglyceride lowering drugs that will reduce the risks of atherosclerosis, respectively.

CHAPTER 6

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